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MICROPROPAGATION IN ELITE CORK OAK TREES: A TOOL FOR SUBER PRODUCTION IMPROVEMENT?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Conceição Santos, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Professor Doutor Marc De Loose, Director de Investigação do Instituto de Investigação para Agricultura e Pescas, Gent, Bélgica.

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Dedico este trabalho à minha mãe Edite…

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palavras-chave

ABA, AFLP, ciclo celular, citometria de fluxo, embriogénese somática repetitiva, embriogénese somática secundária, embriogénese somática, estabilidade genética, genótipo, histo-citologia, luz, maturação, PEG, RAPD, reguladores de crescimento, sobreiro, SSR, temperatura.

resumo

Dada a extrema importância económica e ambiental que o montado de sobro tem em Portugal, e dado o declínio deste devido a várias razões (e.g. doença, idade das plantas) é premente desenvolver estratégias de preservação de sobreiros elite e optimizar técnicas para a propagação destes genótipos.

No primeiro Capítulo expõe-se uma breve introdução sobre o montado actual e as técnicas actuais de regeneração/propagação do sobreiro. Descreve-se ainda as principais técnicas de preservação e avaliação de estabilidade genética referidas na literatura para sobreiro e outras lenhosas.

No Capítulo II é apresentado um estudo de melhoramento das condições actuais de maturação de embriões somáticos de sobreiro com vista a aperfeiçoar o processo de conversão em plantas. Neste capítulo é apresentado um protocolo melhorado em relação ao actual que permite um desenvolvimento dos embriões somáticos dum modo semelhante aos embriões zigóticos em termos de substâncias de reserva.

O Capítulo III mostra um estudo efectuado com o objectivo principal de avaliar estabilidade genética durante todo o processo de embriogénese somática. Neste capítulo são apresentados resultados duma análise feita por RAPD em fases distintas da embriogénese somática de sobreiro. Neste estudo mostra-se que não existem diferenças significativas entre plantas de campo, embriões somáticos e plantas regeneradas.

No Capítulo VI, pretende-se complementar o estudo anterior. Neste Capítulo descreve-se a dinâmica do ciclo celular durante as primeiras fases de embriogénese somática na presença de reguladores de crescimento. Este trabalho permitiu concluir a importância dos reguladores de crescimento na indução e perceber o peso do factor genótipo durante o processo.

Considerando os resultados anteriores, a necessidade de um processo eficiente de preservação de genótipos elite torna-se fundamental. No Capítulo V descreve-se um protocolo de criopreservação eficiente sem recursos a substâncias tóxicas. Nesta secção é ainda feita uma análise de variabilidade genética após criopreservação através de FCM, AFLP e SSR.

Todos os resultados obtidos anteriormente são postos a prova no Capítulo VI onde se faz uma monitorização extensiva de 10 genótipos elite, tendo em conta a sua capacidade de produção de cortiça, através do processo de embriogénese somática. Durante esta secção são utilizados os protocolos desenvolvidos anteriormente e avaliados na sua eficiência. Neste capítulo é descrita a integração de vários segmentos deste estudo num só protocolo eficiente de regeneração e preservação de sobreiros através de embriogénese somática.

Finalmente, no Capítulo VI são apresentadas as conclusões da presente Tese de Doutoramento, com especial incidência para linhas de investigação futuras a serem tomadas. Discute-se a importância deste novo protocolo na optimização da produção da cortiça e traçam-se possíveis aplicações alternativas.

keywords

ABA, AFLP, cell cycle, cork oak, flow cytometry, genetic stability, genotype, histocytology, light, maturation, PEG, plant growth regulators, RAPD, repetitive somatic embryogenesis, secondary somatic embryogenesis, somatic embryogenesis, SSR, temperature.

abstract

Given the extreme economic and environmental importance that cork forests have in Portugal, and because of their decline due to various reasons (e.g. disease, age of plants) is urgent to develop conservation strategies of elite oaks and optimize techniques for the propagation of these genotypes.

In the first Chapter a brief introduction to cork oak and current techniques for its regeneration/propagation are presented. It also describes the main techniques of preservation and evaluation of genetic stability referred in literature for cork oak and other hardwood plants.

Chapter II presents a study to improve the current conditions for maturing cork oak somatic embryos in order to improve the conversion process to plants. This chapter presents an improved protocol that allows a development of somatic embryos in a way similar to zygotic ones in terms of reserve substances.

In Chapter III it's presented a study conducted with the primary objective to assess the genetic stability during the whole process of somatic embryogenesis. This chapter shows results of an analysis by RAPD in distinct phases of somatic embryogenesis of oak. This study reports no significant differences between plants of the field, somatic embryos and regenerated plants.

In Chapter VI, it is intended to complement the previous study. In this chapter we describe the dynamics of cell cycle during the early stages of somatic embryogenesis in the presence of growth regulators. This study revealed the importance of growth regulators on the induction and demonstrated the weight of genotype factor in the process.

Considering the previous results, the need for an efficient process for the preservation of elite genotypes is fundamental. In Chapter V we describe an efficient cryopreservation protocol without using toxic substances. This section also presents an analysis of genetic variability after cryopreservation by FCM, SSR and AFLP.

All results obtained above are put to the test in Chapter VI where there is extensive monitoring of 10 elite genotypes, taking into account their ability to produce cork, through the process of somatic embryogenesis. During this section the protocols previously developed are used and evaluated for their efficiency. This chapter describes the integration of various segments of this study into one efficient protocol for regeneration and preservation of oak trees through somatic embryogenesis.

Finally, Chapter VI presents the conclusions of this PhD thesis, with special reference to future lines of investigation to be followed. It discusses the importance of this new protocol on optimizing the production of cork and draw up potential applications.

"The individual has always had to struggle to keep from being overwhelmed by the tribe. If you try it, you will be lonely often, and sometimes frightened. But no price is too high to pay for the privilege of owning yourself."

Friedrich Nietzsche

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Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid
ABA: Abscisic acid
AFLP: Amplified Fragment Length Polymorphism
BAP: 6-benzylaminopurine
bp: base pairs
CRY25: Cryopreserved tissue after dehydration to 25%
CRY35: Cryopreserved tissue after dehydration to 35%
CV: coefficient of variation
EC: Embryogenic culture
EDTA: Ethylenediamine tetraacetic acid
FCM: Flow cytometry
FPCV: full peak coefficient of variation
FW: Fresh weight
IAA: indole-3-acetic acid
IBA: Indol-3-butyric acid
Log: logarithmic
MS: Murashige and Skoog culture medium
MSWH: solid MS medium without hormones
NAA: 1-Naphthaleneacetic acid
NEC: Non-embryogenic culture
PAS: Periodic acid-Schiff
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PEG: Polyethylene glycol
pg: picograms
PGR: Plant growth regulator
PI: propidium iodide
PVS2: plant vitrification solution 2
RAM: root apical meristem
RAPD: Random Amplification of Polymorphic DNA

RFLP: Restriction fragment length polymorphism

SAM: shoot apical meristem

SE: Somatic embryogenesis

SSE: Secondary somatic embryogenesis

SSR: Simple sequence repeats

TRIS: tris-(hydroxymethyl)-aminomethane

WC: Water content

WPM: woody plant medium

Chapter I

Cork oak propagation – a general introduction

Chapter partially submitted as a revision in a non indexed international journal:

Pinto G, Loureiro J, Costa A, **Fernandes P**, Santos C (2011) New plants for Cork Oak Landscapes: Micropropagation and genetic stability studies. *In* Aswani Kumar and Sudhij K. Sopory Editors. *Plant Tissue Culture and Applied Plant Biotechnology*. 6: 130-152

Cork Oak: ecology, distribution and socio-economic magnitude

Cork oak (*Quercus suber* L.) belongs to the genus *Quercus* that comprises more than 600 species, most of them characterised by their fruits (acorns). It is an evergreen tree, up to 20 m height which sometimes reaches a diameter over 1.5 m, with downy twigs, rather sparse leaf canopy, and a very thick and deeply ridged bark. The cork is a product of a secondary meristem and its extraction takes place when the individual tree reaches an age of 25-30 years. From that point onwards, the bark is stripped every 10-15 years, due to the tree's ability to produce a new meristem once the bark is removed. Cork is mainly used as beverage sealant and insulation material (Gonçalves, 2000).

The worldwide distribution of the cork oak is confined to the centre and western Mediterranean basin, including Portugal, Spain, France, Italy and North Africa. Within Mediterranean ecosystems, cork oak presents key ecological and socio-economic roles. The international importance of the species is well recognized, being a target of the operating gene conservation network of the European Forest Genetic Resources Programme, coordinated by the International Plant Genetic Resources Institute (IPGRI) and FAO (Vogiatzakis and Careddu, 2003). Portugal is the leading producer of cork with 55% of world's production, followed by Spain with 26 % (Toribio *et al.,* 2005). In Portugal, in the last few years, cork oak has suffered a severe decline, especially during drought periods. Research studies targeted to identify the causes of such decrease, showed positive correlations between general decline symptoms and variables like overexploitation, stand ageing without natural regeneration, drought, soil characteristics and anthropogenic stress. It was concluded that the multiple uses of traditional cork oak landscapes contributed to forest decay by making it more sensitive to particular climate occurrences (Ferreira, 2000).

Conventional practices of cork oak propagation

Traditionally, cork oak propagation has been done using the seeds (Bellarosa, 1991; Chalupa, 1995; Bueno *et al.,* 2000). Despite there are usually no major problems with seed's germination, forest regeneration programmes of this species are severely constrained by grazing. Furthermore, acorn production is irregular and highly dependent on climatic conditions (Bueno *et al.,* 2000) and cork oak seeds tend to lose germinating capability after a certain storage period (Chalupa, 1995). This species presents high heterozygocity, caused not only by its allogamy, but also by possible past hybridizations with other species of the genus *Quercus* (Natividade, 1990). Moreover, plants obtained by seed propagation, are genetically

unpredictable, and considering the long duration of the juvenile stage (approximately 25 years), only when the plant reaches the adult stage it is possible to evaluate interest traits (Chalupa, 1995). Therefore, these features limit the establishment of seed orchards and its use in genetic improvement programs, contrarily to what is currently done for some forest species (Celestino *et al.,* 2005; Pinto *et al.,* 2008).

The high heterozygocity referred above together with the fact that most of the interest traits are related with the non-additive genetic component of the genetic variance, result that only through vegetative propagation it is possible to perpetuate the trees with valuable interest (Pinto *et al.*, 2008). Furthermore, since selection of elite cork oaks is only possible in adult plants, the use of some of the traditional techniques of vegetative propagation to provide a reliable alternative to propagation via seed and a means of capturing genetic gains becomes more difficult. Research on conventional vegetative propagation methods has been followed since a long time, but with little success (Natividade, 1990; Bueno *et al.,* 2000). Therefore, and considering the projection of climate change scenario for Mediterranean region (IPCC, 2007) it is of utmost importance to introduce more adequate non-conventional methodologies for cork oak propagation and breeding.

Facing this scenario, forestry practices, especially those directed to the selection and propagation of elite trees may profit from the current technological advances. Research areas such as micropropagation, somatic embryogenesis, genetic engineering, marker-aided selection and molecular diagnostics, are being merged with traditional studies, to help in the identification and production of better-suited trees for forestry plantings. Also, a combination of classical and molecular research could be used to improve pest and stress resistance of selected genotypes and monitor pest attacks on trees (Klopfenstein and Kerl, 1995). In order to transfer the benefits of different biotechnologies to forestry plantings, a reliable plant regeneration technique is essential (Celestino *et al.,* 2005). These authors highlight that all these biotechnological tools are of no value if it is not possible to mass propagate clonal plants and obtain healthy individuals (either transgenic or not).

In vitro plant culture

In the last decades, there has been a rapid progress in the development of in vitro culture techniques for the regeneration and clonal propagation of plants of interest (Merkle and Nairn, 2005). These in vitro culture techniques allow the development of different pathways for plant regeneration in a reproducible way (from several genotypes) and from several cell/tissues, resulting in true-to-type individuals (Kumar *et al.,* 2006). The number of forest

trees propagated by in vitro culture techniques, which have been successfully used in forest breeding programs, has increased in the past years (for a review in hardwood tree biotechnology see Merkle and Nairn, 2005). Most of the successful cases refer to conifer species, but recently there have been significant advances in the biotechnology of angiosperm forest trees using these techniques (Merkle and Nairn, 2005).

In general, woody species are more difficult to propagate in vitro than herbaceous ones. This is due to a lower regenerative capacity, to the production and release of toxic compounds into the growth medium and to the extra difficulty of explants sterilization in woody species. Also, the phase change phenomena or ontogenetic aging, may contribute to their recalcitrancy to in vitro propagation. Finally, due to their high heterozygocity, for many woody plants the results are more variable. (Gaj, 2004; Jiménez, 2005).

In the last decades, there have been enormous efforts in optimizing in vitro conditions for oaks, particularly cork oak (e.g. Toribio *et al.,* 1998; Pinto *et al.,* 2002; Hernández *et al.*, 2003a), from induction to acclimatization. Obviously, there are several factors that may affect the regeneration capacity of the explant: factors determined by the donor plant conditions (e.g., age, physiological status, type of explant and its position in the plant, period of collection of plant material and genotype) and those related to environmental conditions of the culture (e.g., composition of culture medium, type and concentration of growth regulators, container volume, quality and quantity of light and temperature) (e.g., Gaj, 2004; Jiménez, 2005).

In the particular case of woody species with industrial interest, there are two main pathways to in vitro propagate large numbers of plants: micropropagation by nodal/axillary cuttings and somatic embryogenesis (SE).

Micropropagation by axillary shoots

The micropropagation by axillary shoots has proved to be an effective methodology in several woody species (e.g., *Populus* sp., *Eucalyptus* sp., *Robinia pseudoacacia*, *Liquidambar styraciflua*, *Prunus* sp., *Juglans* sp., and *Ulmus*; for a review see Merkle and Nairn, 2005). Unfortunately, in cork oak, only few reports were based in this classic micropropagation methodology.

In vitro culture in *Quercus* was subject to major developments in the 1970's and 1980's. Since then it was possible to micropropagate several species of *Quercus*, such as *Quercus robur* (from nodal and apical segments; Chalupa, 1995) and *Quercus rubra* (from nodal segments; Bellarosa, 1989). Concerning, cork oak, the first report dates to 1952, when Jacquiot obtained callus from in vitro cultures of cambial tissue (Jacquiot, 1952 *vide* Bellarosa, 1989). Later,

Bellarosa (1981) obtained subcultures of axillary shoots from in vitro cultures of cork-oak zygotic embryos and Pardos (1981 *vide* Bellarosa, 1989) produced cork oak cultures from nodal segments of 12 month-old seedlings. In 1988, Deidda and co-workers, reported plantlet regeneration from axillary shoots of seedlings (Deidda *et al.*, 1988). However, in three works little success was achieved in the rooting process. The first report of successful micropropagation from adult material is attributed to Manzanera and Pardos (1990) who reported in vitro plantlet multiplication using apical buds and nodal stem segments from different explant sources (young seedlings, stump sprouts and sprouts from old trees). The authors suggested that media with low concentrations of ions, such as Sommer's or Heller's, are more suitable for the growth and proliferation of explants of cork oak (Manzanera and Pardos, 1990). The plant regeneration from adult trees opened the perspective to propagate selected trees with desirable characters. When field material of is used, one of the most important and limitative factors in micropropagating this species are the phytosanitary conditions of the mother plant and the process of disinfection. Although these authors had no problems with contaminants, a high percentage of toxic compounds were released into the growth medium and consequently a large amount of plant material was lost. Later, Romano and Loução (1992) used Gresshof and Doy (1972) medium to initiate cultures from axillary/terminal buds, and were able to obtain similar results to Manzanera and Pardos (1990). However, the difficulties of the micropropagation process, mainly culture browning and bacterial contamination, were highlighted by the authors. By the same time, Gonçalves and Rainho (1992) were able to obtain plantlets from axillary shoots of 4-9 month old cork oak after inoculation on MS medium (Murashige and Skoog**,** 1962). More recently El-Kbiach *et al.* (2004) tested different macronutrient formulas and proposed that the combination of MS micronutrients and WPM macronutrients improved caulogenesis and shoot multiplication. Furthermore, several studies have already highlighted the importance of cytokinins (e.g., BAP: 6-benzylaminopurine) in culture development (Manzanera and Pardos 1990; Pinto 2002) and the relevance of adding low levels of auxins (e.g., NAA: naphthaleneacetic acid) to the medium to improve shoot multiplication, especially in vegetative material of adult origin (Manzanera and Pardos 1990).

After *in vitro* culture establishment, shoot proliferation and elongation, the next critical stage is rooting. In this stage, the type of auxin is fundamental to promote rhizogenesis. In cork oak, continuous exposure of low concentrations of IBA (Indole-3-butyric acid) or the dipping of the base of the shoots in a concentrated solution of IBA gave the best rooting results (Manzanera and Pardos, 1990; El Kbiach *et al.*, 2004; respectively).

Other factors, such as carbon source, also affect the rates of cork oak shoot proliferation and in vitro rooting (Romano *et al.,* 1995). While sucrose (3%) allowed the best shoot elongation rates, enabling an effectively higher number of shoots during media transfer, 4% glucose was the best carbon source during rooting phases. Romano and Loução (2003a) also highlighted the importance of darkness during the first week of rooting which resulted in a remarkable enhancement of the rooting percentage, number of roots developed per shoot and length of the longest root.

In a more conservation oriented study, Romano and Loução (1999) described a simple system for in vitro conservation of cork oak shoot cultures. The authors were able to store cork oak shoots *in vitro* on a multiplication medium (Gresshof and Doy medium containing 1 mg I^{-1} of BAP) at 5 ± 1°C, without any further subculture, for two years at dark conditions. These authors also suggested that this procedure could be a promising technique for medium-term conservation of cork oak's germplasm.

Considering the acclimatization phase, the available information is scarce. Romano and Loução (2003a) reported the acclimatization of cork oak well-rooted plantlets in a mixture of peat and vermiculite and incubation in a growth chamber with high humidity. Hardening of plantlets under high humidity during four weeks was found to be essential for successful acclimatization. After two months, plants were transferred to the glasshouse and the percentage of surviving plantlets after six months ranged from 60% to 72%. In a similar strategy, El-Kbiash *et al.,* (2004) 92% reported that rooted plants were successfully potted on horticultural substrate and incubated in a chamber with a high relative humidity.

Regarding the performance of cork oak micropropagated plantlets after transference to ex vitro conditions, the scenario is not different and to our knowledge only one study has been performed. Romano and Loução (2003b) characterised some aspects of the foliar anatomy of cork oak, and measured the rates of water loss by different types of leaves during the acclimatization period. The results are very similar to those reported for other species where acclimatization was followed (for a review see Hazarica, 2006). Leaves of in vitro grown plants presented open stomata and collapsed guard cells, while acclimatized leaves presented closed stomata. Also, a shade-leaf structure with large intercellular air spaces and a low mesophyll cell density, but with a differentiated palisade cell layer was observed in transverse sections of in vitro leaves. Leaves from acclimatized plants showed a sun-leaf structure with small intercellular air spaces, high cell density and two or three palisade cell layers. During acclimatization, leaf thickness increased, as well as, cell compactness and differentiation. Stomatal density, aperture and guard cell protuberance decreased during the acclimatization period, while trichome density increased (Romano and Loução 2003b).

Somatic embryogenesis: a powerful tool in forest biotechnology

Somatic embryogenesis (SE) is the process by which somatic cells differentiate into somatic embryos. Somatic embryos morphologically resemble zygotic embryos. They are bipolar and bear typical embryonic organs, the radicle, hypocotyl and cotyledons. However, they develop through a different pathway (Merkle *et al.,* 1995; Dodeman *et al.,* 1997; von Arnold *et al.,* 2002). Compared with other in vitro propagation methods, somatic embryogenesis offers several advantages. A separate rooting step is not required, as somatic embryos have both a shoot and a root meristem; SE usually form propagules faster and in much larger numbers per explant; the SE process can be automated, bearing the potential to become cheaper than other clonal propagation techniques currently in use (Park *et al.,* 1998a); and it allows the cryopreservation of the embryogenic clonal lines, while the trees obtained from these lines are tested in the field (e.g., Park 2002). This last point is of great importance for advanced breeding programs and commercial forestry as they can directly benefit from the use of elite clones. In the area of forest biotechnology, this propagation method is nowadays regarded as a system of choice for genetic manipulations and mass propagation of superior forest tree genotypes.

The induction of SE in the *Quercus* genus dates already to 1982, when Srivastava and Steinhauer reported the obtainment of SE from zygotic embryos in *Quercus lebani* (Srivastava and Steinhauer, 1982). After this work, several others in different species followed, most of which using the same source material: *Quercus cautisima* (Kim *et al.,* 1997), *Q. alba* (Gingas and Lineberger 1989), *Q. rubra* (Rancillac *et al.,* 1996), *Q. serrata* (Sasamoto and Hosoi 1989), *Q. pubescens* (Féraud-Keller *et al.*, 1989), *Q. cerris* (Ostrolucka and Petronova 1991), *Q. petraea* (Jörgensen 1993; Chalupa, 1995), *Q. robur* (Chalupa 1995; Manzanera *et al.,* 1996), *Q. canariensis* (Bueno *et al.,* 1996) and *Q. ilex* (Féraud-Keller and Espagnac 1989; Mauri and Manzanera 2003). In the particular case of *Quercus suber*, the majority of the studies also refer to the induction of SE from zygotic or very young material. Induction of SE from immature/young tissues was achieved from: cotyledons of mature zygotic embryos (Toribio 1986), nodal segments (El Maâtaoui and Espagnac 1987; El Maâtaoui *et al.,* 1990), immature zygotic embryos (Bueno *et al.,* 1992; Manzanera *et al.,* 1993) and leaves of young plants (Fernández- Guijarro *et al.,* 1991; Fernández-Guijarro *et al.,* 1995). Despite the lack of knowledge about the future value of plants obtained from zygotic embryos these protocols are a good starting point for future studies using adult recalcitrant material (Merkle *et al.,* 1997).

Where are we standing in the case of Cork Oak

Since the last reviews of Chalupa (1995), Manzanera *et al.* (1996) and Wilhelm *et al.* (2000), considerable progress has been achieved in the micropropagation through SE in the *Quercus* genus, especially because reproducible protocols using mature tissues have been finally obtained.

Many differences can be noticed when comparing the protocols for SE initiation from juvenile tissue with those using adult material. The most blatant difference concerns the success rates of the induction stage: while in juvenile tissues it is common to obtain a 100% induction frequency, using mature tissues the success is much lower.

The preliminary studies published by Fernández Guijarro (1997) and Toribio *et al.* (2000) showed the possibility of inducing somatic embryos from leaves of mature cork oak trees. This has been further confirmed by Pinto *et al.* (2002), Hernández *et al.,* (2003a), Toribio *et al.* (2004, 2005) and Hernández (2007), who were able to clonally propagate and obtain emblings (SE-derived plants) from different selected trees. It is also possible to induce SE from floral structures, but in this case it is important to distinguish between somatic and gametic embryogenesis. Bueno *et al.* (1997) were able to produce haploid *Q. suber* plants from anthers by combining a starvation treatment with a mild heat shock at 33 ºC for 5 days, followed by culture at 25 ºC in a simple agar medium without plant growth regulators (PGRs).

Somatic embryogenesis induction

The type of explants, the genotype, the culture medium and the growth regulators have a major influence in the induction of embryogenic cultures and plant conversion. The stress caused by growing conditions may favour the initiation of the embryogenic response by triggering the appropriate signal for genetic reprogramming and expression of totipotency of the somatic cells (von Arnold *et al.,* 2002; Fehér *et al.,* 2003; Jimenez, 2005).

In preliminary studies of SE induction in cork oak (Toribio *et al.,* 2000; Hernandez *et al.,* 2001), it was observed that the basic protocol developed to induce SE from leaves of young seedlings (Fernandez-Guijarro *et al.,* 1995) was also suitable for obtaining embryogenic lines from leaves of adult cork oak trees. However, the induction frequency was low and not all the tested genotypes formed somatic embryos. These protocols were optimized by Hernandez *et al.* (2003a), who were able to induce SE in several genotype from expanding leaves of epicormic shoots after those having been forced to sprout from segments of branches (in a

growth chamber) collected from several hundred years-old cork oak trees. However, contamination was common problem, despite of the highly controlled conditions. This drawback was a matter of study by Toribio *et al.* (2005), who revealed that the putative endogenous contamination is influenced by genotype, collection date and, mainly, by the time of harvesting, with leaves harvested from recently sprouted shoots becoming less contaminated than leaves collected from older epicormic shoots. A major step forward has been achieved by Pinto *et al.* (2002), who was able to successfully induce SE from leaves of mature trees collected directly from the field.

Although there are different protocols available to induce SE from mature leaves (mostly differing in medium composition and the type of plant growth regulators (PGRs)) they share some similarities, namely, the necessity for both an auxin and a cytokinin to induce SE process. The most widely used auxin sources are NAA or 2,4-D (2,4-dichlorophenoxyacetic acid), while BAP, or zeatin are the most frequently used cytokinins. Pinto *et al.* (2002) reported SE in leaf explant calluses obtained from a 60-year-old tree and induced on MS medium supplemented with 4.5 μM 2,4-D and 9.0 μM zeatin, 3% sucrose, 0.3% gelrite. Later on, Hernandez *et al.* (2003a) suggested the induction of SE following a two-stage method (as described in Fernandez-Guijarro *et al.* 1995), but with an extra preconditioning in vitro phase. The culture medium used at preconditioning consisted of Gamborg's macronutrients diluted by half, Murashige and Skoog (1962) (MS) micronutrients, vitamins and Fe-EDTA, supplemented with 1% sucrose and 0.6% agar, (without PGRs). The primary medium for induction consisted of Schenk and Hildebrandt (1972) macronutrients, MS micronutrients, vitamins and Fe-EDTA, supplemented with 3% sucrose, 0.6% agar, and 10 μM BAP and 50 μM NAA. The secondary medium for induction had the same composition as the primary one, but the concentrations of PGRs were reduced to 0.5 μM BAP and 0.5 μM NAA. Furthermore, these authors verified that SE induction frequency was significantly higher when the exposure to growth regulators was increased from seven to 30 days and that only in expanding leaves with less than 15 mm length it was possible to induce somatic embryos suggesting the existence of a development window that could be more prone to induction. In these protocols, a period in the dark of approximately 30 days, after which the cultures were transferred to the light, was reported (Pinto *et al.,* 2002; Hérnandez *et al.,* 2003a). Wilhlem *et al.* (2002) highlighted the required period for somatic embryos formation was shorter when juvenile tissues were used as explants in comparison to mature explants. Pinto *et al.* (2002) reported a 3-months period to observe embryogenic structures, whereas Hérnandez *et al.* (2003a) reported SE after 2-months in culture. Later, Toribio *et al.,* (2005) published detailed and reliable protocols for complete plant regeneration of cork oak by SE.

The differences in SE performance among genotypes support a genotypic effect as suggested by Merkle *et al.* (1997). The influence of genotype on morphogenic processes is well documented, including the genetic control of SE induced in zygotic embryos in other woody plants (Park *et al.,* 2002; Lopes *et al.,* 2006; Pinto *et al.,* 2008). Significant variances in the embryogenic response due to the cork oak family influence were detected by Fernandez-Guijarro *et al.* (1996). Toribio *et al.* (1998) and Hernández *et al.* (2003a) revealed a significant influence of individual adult cork oak trees on SE initiation frequency. Fernandez-Guijarro (1997) highlighted that some phases of this process show an additive genetic control, particularly the induction of SE in zygotic embryos, being amenable to genetic improvement.

Independently of the explant source (leaves or zygotic embryos), cork oak somatic embryos show the same developmental pattern. Somatic embryos appeared almost directly on the surface of leaves, without a defined pattern of organization, and they soon started to show secondary embryogenesis The full expression of embryogenesis occurred when the leaves were transferred to PGR-free medium, in which more leaves showed their embryogenic ability, and somatic embryos their potential to multiply themselves by repetitive embryogenesis (e.g., Pinto *et al.,* 2002, Hérnandez *et al.,* 2003a; Toribio, 2005; Hérnandez, 2007).

Repetitive somatic embryogenesis

In dicotyledonous woody species as cork oak, repetitive embryogenesis (SE) is a phenomenon whereby new somatic embryos are initiated from other somatic embryos (Toribio 2005). The process of SE is normally not synchronized (Toribio 2005), therefore, a broad spectrum of different developmental stages can be found simultaneously. In an optimized procedure, this phenomenon can be automated by the use of liquid culture and bioreactors (Park 2002). In cork oak, secondary embryogenesis takes place continuously on media without PGRs (merely by monthly subculture to fresh medium), giving a recurrent process (repetitive SE) that can last for years without an apparent decline of multiplication ability. Additionally, similarly to what occurs in other species, the primary SE in *Q. suber* is less efficient than the secondary SE (Pinto *et al.,* 2002; Hernández *et al.,* 2003a; Pinto *et al.,* 2008b). Other relevant feature of this process is that embryos may reach their full cotyledonar stage without any specific treatment of differentiation, allowing its easy isolation, with new embryos being formed from previous somatic embryos mostly following a multicellular budding pattern (Toribio *et al.,* 2005; Hernández, 2007). Hérnandez *et al.*

(2003a) reported that the initially induced embryos multiplied themselves (repetitive SE) on a MS medium lacking PGRs for more than 2 years without a decline and highlighted that, from a practical point of view, the number of somatic embryos produced per leaf of mature trees, and even the frequency of induction, are not relevant features of the protocol, considering the high rate of proliferation that is further achieved by repetitive SE. Fernandes *et al.* (2008) reported a five years period of maintenance of the embryogenic lines initially induced by Pinto *et al.,* (2002). These oak embryogenic cultures could be stored at low temperature for four months without loss of their proliferative ability, which permitted to reduce the maintenance costs by increasing the gaps between subculture periods.

In cork oak, most of the secondary embryos are formed next to the root pole, and more rarely in the cotyledons (El Maâtaoui *et al.,* 1990; Bueno *et al.,* 1992; Fernandez-Guijarro *et al.,* 1995; Puigderrajols *et al.,* 1996; Bueno *et al.,* 2000; Pinto *et al.* 2002; Hernández *et al.,* 2003a). This fact seems to be a common feature in the *Quercus* genus (Wilhelm 2000). In general, a typical somatic embryo at the cotyledonar stage of cork oak is a bipolar structure flanked by two cotyledons that may appear to have a transparent to opaque appearance according to the degree of maturation (Fernandez- Guijarro *et al.,* 1995; Pinto *et al.,* 2002; Hernández *et al.,* 2003a). The occurrence of abnormal morphologies in the somatic embryos, such as the presence of one or more than two cotyledons or even fused embryos or merged cotyledons, have been frequently reported in this species (e.g., Puigderrajols *et al.,* 1996; Pinto *et al.,* 2002; Hernández 2003b). However, such occurrence, was not reflected at the next stages of the SE process, as the plants derived from these SE showed no morphological variability when compared with those obtained from conversion of normal dicotyledonary embryos (Pinto *et al.,* 2002). Few studies have dealt with the genetic control of the repetitive embryogenesis process and similarly to the induction phase this factor seems to play an important role in secondary SE. Hernández *et al.* (2003b) referred that when embryogenic masses were broken into small pieces and sub-cultured, their growth rate, measured as the increase in relative fresh weight, was very high and strongly influenced by genotype, which explained 21% of the total variance. The genotype also affected the number of detachable embryos produced by the embryogenic clusters, accounting for 14% of the total variance.

There are same controversies concerning the direct or indirect origin of secondary somatic embryogenesis in cork oak. El Maâtaoui *et al.* (1990) did not recognize the presence of callus prior to the appearance of secondary somatic embryos and, thus considered secondary somatic embryos as having a direct origin. Contrarily, Puigderrajols *et al.* (1996) referred that secondary SE has an indirect origin since it occurs after an initial proliferation of callus on the area next to the root pole. In support of the latter view Pinto *et al.* (2002) observed the

formation of a mass next to the root pole of initially isolated embryos, precisely in the area where later on the secondary embryos appeared. Ultrastructural studies of the secondary SE process revealed the occurrence of both pathways: the multicellular pathway from a compact mass of proliferation and the unicellular pathway from isolated cells in friable callus (Puigderrajols *et al.* 2001). The origin of these secondary somatic embryos is extremely relevant to the genetic uniformity of regenerated plants, as a multicellular origin may result in the formation of genetically variable plants (chimeras). A unicellular origin is undoubtedly the most desirable pathway for applications that involve the cloning of SE, such as genetic transformation. The protocols should be optimized in order to synchronise the SE process and promote the unicellular pathway of regeneration in favour of the multicellular one (Puigderrajols *et al.,* 1996, 2001).

Maturation of somatic embryos

Has highlighted above, the recurrent secondary embryogenesis is the basis of the great potential of this technique for mass clonal production of somatic embryos. However, if the production of individual plants is desired, this process has to be arrested and the individual embryos should be left to mature (Toribio *et al.*, 2005).

Maturation may, sometimes, occur spontaneously by the end of the subculture period. A low availability of nutrients and consequently some degree of starvation have been pointed as the inducing factors of spontaneous maturation in cork oak (Toribio *et al.*, 2005). In this case secondary embryogenesis ceases and somatic embryos enlarge and become white opaque. These somatic embryos can then be selected for the germination/conversion phase (Toribio *et al.,* 2005). However, to use SE as a mass vegetative propagation technique of high value trees for forest planting, this maturation stage needs to be controlled and improved.

Abscisic acid (ABA) is a well-known PGR involved in the maturation process, promoting the accumulation of reserve substances, and reducing the frequency of abnormal morphologies and secondary embryogenesis (Gaj 2004). In cork oak, some experiments aimed to control the recurrent cycle by the addition ABA to the culture medium were inconclusive, i.e., no visible effects of ABA on the arrest of repetitive embryogenesis were detected (Pinto *et al.,* 2002; Toribio *et al.,* 2005). However, other studies, suggested that the addition of ABA was important to promote SE maturation (Bueno *et al.*, 1992; Garcia Martin *et al.*, 2005). Garcia Martin *et al.* (2005) found that the addition of 1 µM ABA to the culture medium promoted somatic embryo maturation and increased both fresh and dry matter, without affecting the relative water content. However, these works differed from the formers, as the source

explants for SE induction were leaves of a 2-month old *Q. suber* seedling. Alternative strategies for SE maturation include partial desiccation under high humidity conditions (Toribio *et al.,* 2005) and starvation treatments (Fernandez-Guijarro *et al.* 1994, 1995). In detail, the latter strategy consisted on an alternate culture of somatic embryos on (i) a medium that increased embryo proliferation and (ii) a low salt medium inhibiting embryo formation, which would partially synchronize embryo development. Moreover, maturation under light followed by storage at 4 °C for at least 30 days was found to be very important in switching embryos from an embryogenic pathway to a germinative one. Under these conditions 15% of the embryos showed coordinated root and shoot growth and 35% formed either shoots or roots (Fernandez-Guijarro *et al.,* 1995). Unfortunately, few studies provided detailed data on the effect of maturation treatments on the subsequent steps of germination and conversion.

Germination and conversion of somatic embryos

One of the main problems of cork oak regeneration by SE is the low rates of somatic embryos conversion into plants. This low conversion is common in the genus (Wilhelm 2002) and, as referred by Chalupa (1995), it is common that the development of SE is blocked after the formation of the cotyledons, which may acquire a green colour, but do not proceed into the next developmental stage. According to Fernandez-Guijarro *et al.,* (1995) from the moment that somatic embryos are programmed to origin more embryos, the normal development is blocked, and only after this programme is interrupted, somatic embryos can proceed to germination.

Cold is considered the most efficient treatment to trigger the conversion process, being widely used, not only in *Quercus* but also in conifers (Haggman *et al.,* 1999). In cork oak, several studies used this strategy to promote germination and conversion of somatic embryos (Bueno *et al.,* 1992; Manzanera *et al.,* 1993; Fernandez-Guijarro *et al.,* 1995; García-Martin *et al.,* 2001; Pinto, 2002). In the particular case of Garcia-Martin *et al.* (2001), improved germination rates (best results close to 100%) were obtained not only by chilling somatic embryos for two months, but also by increasing the sucrose concentration on the culture medium to high levels (15%). In Pinto (2002), it was referred that cold significantly increased embryo conversion and decreased the number of secondary somatic embryos formed per initial explant, and that germination and/or conversion mainly occurred in embryos in a more advanced stage of evolution. Concerning the use of sucrose, it is suggested that the lack of sufficient reserves in the cotyledons to support the advanced stages of

germination, mainly the growth of a large tap root before leaves are functional, may be responsible for the low percentages of conversion that are usually reported.

After germination, González-Benito *et al.* (2002) suggested that a low concentration of BAP (0.04 μM) allowed an appropriate radicle elongation in all germinating somatic embryos, and that higher concentrations of this PGR has opposite effects. It was also verified that the application of BAP increased caulinar apex elongation and maintained an active growth of the plantlets, although no significant effect on the percentage of shoots with normal morphology was observed. However, once again, genotype affected all the variables recorded during conversion.

Plantlet acclimatization

At present, cork oak in vitro plant regeneration is well developed to fulfil low-level demands (e.g., conservation purposes, genetic assays, and plant development studies), as only a limited number of plants are usually obtained (Toribio *et al.,* 2005). This results not only from the problems verified in the stages mentioned above (e.g. maturation, germination/conversion), but also from the difficulty of the acclimatization step, in which many plantlets are lost. In general, in vitro conditions contribute to the formation of emblings with physiological and anatomical characteristics that need to be gradually acclimatized to greenhouse and/or field environments (for review see Hazarica 2006). Since the last revision in *Quercus* (Wilhelm 2000), considerable efforts have been made to optimize plant regeneration in oaks, but the process of acclimatization to the soil substrate still remains the major bottleneck for large scale production.

In cork oak, Bueno *et al.* (1992) reported that thirty-one plantlets developed normally and were transferred to soil and acclimated to greenhouse conditions, but no details on the survival rates were given. Manjon *et al.* (1998) reported that when *Q. suber* emblings were transferred to substrate they stop growing, and after some time died. In search of a solution to these problems, Diez *et al.* (2000) studied mycorrhizal associations in cork oak and observed that in vitro mycorrhization with *Scleroderma polyrhizum* and *Pisolithus tinctorius* increased the formation of secondary roots and the survival rate after acclimatization of plants obtained from somatic embryos. Later on, Hernández *et al.* (2003b) referred that after transferring the plantlets to ex vitro conditions bearing high relative humidity conditions, nearly half of them ceased growing and, after a decrease in relative humidity, 67% died. Only 33% of the plants that survived the change to low humidity were completely acclimatized.

This means that, in the whole conversion process and depending on the genotype, only a few of the somatic embryos reached the state of completely acclimatised plants.

A recent strategy of acclimatization consists in transplanting the emblings to 180 ml forest containers, filled with substrate (pine bark:peat:sand, 3:1:1, v/v/v) and designed to avoid malformations in root growth, under ex vitro conditions. These emblings were placed in a growth chamber and were covered with inverted glass beakers to guarantee a high humidity. After two months, the beakers were removed 1 h/day during one month. Finally, the plants were moved to a nursery, under shade conditions (Hernández *et al.,* 2003b; Toribio *et al.,* 2005).

Most of the studies that reported emblings regeneration and acclimatization just provide the number of surviving plants, and only a few works gave details about their morphology and field performance. The ability to induce SE from leaves of adult trees opens the possibility to compare the performance of the parent trees and their progenies at the same age and place of test. Celestino *et al.* (2007) reported a field test using emblings regenerated from five selected cork oak trees and from young plants of their half-sib progenies. They were planted in the field together with zygotic seedlings of these families, and the performance of plantlets of somatic vs. zygotic origin and plantlets obtained from SE of mature trees vs. SE of juvenile seedlings was compared. The effect of genotype and kind of progeny were studied using a complete factorial design; therefore the field trial comprised 15 treatments arranged in five completely randomised blocks. The authors observed that all the plants originated from seeds survived, while almost half of somatic seedlings died after the winter and needed to be replaced. Almost 70% of these plants survived after the following summer. There were no differences between somatic seedlings of mature or juvenile origin. Zygotic seedlings doubled their height relatively to the somatic ones after one year in the field, and, within somatic seedlings, those of mature origin grew slightly more than those originated from juvenile explants. Also, no apparent morphological alterations were detected among the three kinds of progenies. However, no complementary studies at the physiological level were performed that could sustain the obtained results. This is indeed, an important gap in the current knowledge of the acclimatization process in cork oak.

To improve this propagation system and overcome its main constraints, particularly the maturation stage and the low germination frequency, it will be fundamental to increase our knowledge in the developmental physiology aspects governing the SE production process. In order to optimize these and the further steps, one should focus on comparative physiological, biochemical and molecular studies of both the zygotic and somatic embryos development. As referred by Wilhelm (2000), due to the limited data available on plant performance, many

questions cannot be answered and more studies concerning plant physiology during acclimatization and field trials are needed.

Evaluation of genetic fidelity

The commercial use of any micropropagation technique implies the assessment of emblings performance in the field and the analysis of their genetic stability (Park 1998). The economic consequences of somaclonal variation can be enormous in forest trees, due to their long life cycles. Therefore, plants presenting somaclonal variation should be detected and eliminated from the subsequent stages of the breeding program, in order to guarantee the success and acceptance of a large-scale micropropagation scheme of a particular plant species.

Somatic embryogenesis has often been regarded as a stable system; however, as referred above and as it occurs in other *Quercus* species, there is the chance that cork oak SE has a multicellular origin that increases the risk for somaclonal variation (Wilhelm, 2000). Reports in *Q. robur* (Wilhelm *et al.,* 1999; Endemann *et al.,* 2001) demonstrated that several years of continuous culture can result in tetraploidy. In cork oak, the possibility to propagate this species by secondary embryogenesis in media lacking PGRs minimizes this risk for genetic variation, but still it is important to screen the resulting emblings (at least) and compare them to the parental tree.

Chromosomic mutations like inversion, deletion or translocation and genetic mutations can be detected by molecular markers such as RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms) or microsatellites/SSRs (simple sequence repeats), while major genetic changes, like polyploidy and aneuploidy can be easily screened by the use of flow cytometry.

In *Q. suber*, RAPD markers were used to monitor the genetic stability of somatic embryos (Gallego *et al.,* 1997). In this study no aberration in RAPD banding pattern was found among the tested samples. Hornero *et al.* (2001) suggested that genetic fingerprinting based on amplified fragment length polymorphisms (AFLP) is more powerful and reliable than RAPDs, as it allows the direct analysis of variation at the entire DNA level with the generation of more reproducible markers. In that work, SE was induced in expanding leaves collected from mature cork oak branches that were forced to sprout. DNA was extracted from leaves of the mother plants (three different genotypes) and from somatic embryos derived from each tree. In one tree, AFLP patterns from leaves and somatic embryos were identical, but some variation was detected in somatic embryos from the other two trees. Although the level of

genetic variation detected in these lines is lower than that recorded for half sibs of cork oak, its influence on phenotypic variation needs further assessment (Hornero *et al.,* 2001). More recently, the genetic stability of the SE process was also evaluated by simple sequence repeats (SSRs = microsatellites) (Lopes *et al.,* 2006). Uniform microsatellite patterns were, in general, observed within and between somatic embryos and the respective donor genotypes. For one genotype the same pattern was observed in all the analysed samples, except one, where a mutation was found (accounting for 2.5% of the tested material).

Other methodologies focused on ploidy changes must also be routinely used to assay genetic stability. Due to its precision and rapidity, flow cytometry (FCM) appears as the ideal technique to easily achieve such a goal. However, only a few works have used this technique to assess somaclonal variation in cork oak micropropagation (Bueno *et al.,* 1996; Bueno *et al.,* 2000; Loureiro *et al.,* 2005; Santos *et al.,* 2007). The most complete study so far, showed no major ploidy differences between somatic embryos and the mother plant from which they were obtained as well as ploidy level stability between somatic embryos with two cotyledons (considered as normal) and abnormal ones, presenting one or more than two cotyledons (Loureiro *et al.*, 2005).

Cryopreservation, artificial seeds and genetic transformation

As mentioned above, SE has a great potential for vegetative mass propagation of superior forest tree genotypes. However, the long periods that are needed for evaluating the quality of regenerated material, makes it imperative to store the source cultures for several years (Vendrame *et al.,* 2001). Cryopreservation has been used as a suitable and efficient mean for long term storage of plant tissues and cell cultures, including embryogenic cultures, which proved to be amenable to cryogenic conservation (Park 2002).

Cryopreservation techniques offer several advantages in tissue culture: it reduces labour and supply costs, it decreases the risk of culture contamination, it retains embryogenic capacity by reducing culture deterioration, it limits somaclonal variation and it may enhance the embryogenic capacity by the elimination of highly vacuolated non-embryogenic cells (Vendrame *et al.,* 2001). Currently, cryopreservation was already successful in the conservation of different types of materials, including seeds with orthodox and intermediate storage behaviour, dormant buds, pollen, biotechnology products and apices sampled from *in vitro* plantlets of vegetatively propagated species (Engelmann, 2004). However, considering its high potential, it is expected that cryopreservation will soon become more and more used in the long-term conservation of plant genetic resources.

Shoot-tips and somatic embryos are excellent material for in vitro long-term storage (in liquid nitrogen) of ex situ plant genetic resources. Cryopreservation of organized structures has significantly progressed with the development of several vitrification-based protocols, such as encapsulation-dehydration and PVS2. These approaches allowed significant improvements in the survival and recovery after cryopreservation when compared with conventional crystallization-based protocols (Gonzalez-Arnao *et al.,* 2008).

Among forest species, cryopreservation protocols have been successfully applied to embryogenic cultures of white spruce (*Picea glauca* (Moench) Voss), silver birch (*Betula pendula* Roth) and radiata pine *Pinus radiata* D. Don), among others (for a review see Vendrame *et al.,* 2001). In oaks, there are already several studies that demonstrated the feasibility of the cryopreservation of somatic embryos. González-Benito *et al.* (2002) studied different aspects of the cryopreservation protocols used in *Quercus ilex* and *Q. suber* embryonic axes. In this study, *Q. suber* axes proved to be more sensitive to desiccation and cooling than *Q. ilex*. Only 35% of the axes survived when included into cryovials and immersed directly in liquid nitrogen, while none survived when immersed in sub-cooled liquid nitrogen (-210 °C). Later Valladares *et al.* (2004) described a protocol for cryopreservation of embryogenic lines induced from leaves of adult trees of cork oak. These authors used a simple vitrification procedure, consisting of a pre-culturing of 2-4 mg clumps (with two or three globular embryos) on semisolid medium containing 0.3 M sucrose for three days, followed by incubation in PVS2 vitrification solution at 0C (for 60 minutes) before direct immersion in liquid nitrogen, and were highly successful in the cryopreservation of three cork oak embryogenic lines (88-93% of embryo recovery). The average number of embryos produced per explant was significantly higher for cryostored embryos than for untreated stock cultures and the germination and plant regeneration rates of cultures derived from cryostored embryos, were around 60%, similar to those of non-cryopreserved stock cultures. Recently, Fernandes *et al.* (2008) described a highly successful encapsulation-dehydration procedure for cork oak somatic embryos. During this procedure, embryogenic clusters were encapsulated in an alginate bead, cultured for 3-days in 0.7 M sucrose, desiccated to 25 or 35% water content (WC), and frozen in liquid nitrogen. After thawing, cryopreserved somatic embryos had high viability and exhibited long-term survival (approximately 90% survival). Also, no morphological differences were observed between somatic embryos desiccated to 25 and 35% WC (Fernandes *et al.,* 2008). These authors highlighted that the used method minimizes the risk of tissue injury, because, unlike vitrification, it does not require toxic cryoprotectants as PVS2. As cryopreservation may also induce genetic instability (Sakai 2004), Fernandes and co-workers evaluated the genetic

stability of regenerated material using FCM, AFLPs and SSRs. Flow cytometry confirmed that both ploidy level and DNA content were consistent with the available literature (2C = 1.90 pg DNA; Loureiro *et al.,* 2005; Santos *et al.,* 2007) and that no significant differences between control and cryopreserved samples were detected at the DNA-sequence level.

In sum, the cryopreservation of oak's somatic embryos allows long-term preservation of germplasm until phenotypic traits are evaluated on adult trees under field conditions. However it is important to assure that embryogenic clones are maintained indefinitely in cryogenic storage without any change in genetic makeup or loss of viability (Park 2002). If such conditions are guaranteed, it is possible that in a near future cryopreservation together with the ultimate developments in SE protocols from mature trees are applied in cork oak improvement programs.

Another storage technique is the production of synthetic seeds. The first attempts to determine the optimal storage period and the optimal conditions for conversion of encapsulated somatic embryos of cork oak were published only recently by Bueno *et al.,* (2008). However, these authors used somatic embryos induced from zygotic seeds. The somatic embryos were coated with alginate for the production of synthetic seeds and their storability for commercialization was investigated. Synthetic seeds were cold stored at 4 ºC for two months without significant loss of conversion capacity. The conversion rates of synthetic seeds were higher on a medium supplemented with mineral nutrients than without. The authors referred that this method enabled the assessment of growth parameters without the risk for contamination, which opens the possibility for an automated control of culture growth for the future up scaling of plant production.

Genetic transformation offers an attractive alternative to breeding because it provides the potential to transfer specific traits into selected genotypes without affecting their desirable genetic background. This has particular importance in woody species, since many adaptative and economic traits are under non-additive genetic control and, consequently, a specific genetic make-up has to be transferred to the offspring (Alvarez *et al.*, 2004). A prerequisite for the production of transgenic plants is the availability of a method to regenerate a complete plant from the transformed cell. Cork oak somatic embryo cultures offer an excellent starting point for genetic manipulation. These cultures present low manipulation necessities and high proliferation rates, and the maturation, conversion and acclimatization protocols to obtain plantlets from embryogenic lines are already optimized (e.g. Pinto *et al.,* 2002, Hernandez *et al.,* 2003a), which opens the possibility to manipulate and clone desired genotypes.

The reports available so far are very recent and result from the optimization of the protocols used to regenerate plants by SE. Álvarez *et al.* (2004) developed a protocol to transfer foreign chimeric genes into cork oak. These authors observed that cork oak somatic embryos obtained from recurrent proliferating embryogenic masses were susceptible to AGL1 strain (harbouring the pBINUbiGUSint plasmid,), a disarmed succinamopine-nopaline strain of *Agrobacterium tumefaciens*. Evidence of stable transgene integration was obtained by polymerase chain reaction for the *nptII* and *uidA* genes and by Southern blotting and expression of the *uidA* gene. However, the transformation efficiency (i.e. percentage of inoculated explants producing independent transgenic embryogenic lines) was of 4%, only. The transgenic embryos were then germinated and successfully transferred to soil. Sánchez *et al.* (2005) also presented a protocol for genetic transformation of cork oak using pro-embryo masses induced from immature zygotic embryos in. These embryogenic masses were inoculated with the *A. tumefaciens* LBA4404/p35S GUS INT/pCAMBIA 1301 strain. Transformants were selected on hygromycin supplemented medium, with the viable embryos constituting 13% of the embryos selected on this medium during 4 months. The expression of β-glucuronidase, 4 months after co-cultivation, confirmed a transformation rate of 5.8%. Recently, Álvarez and Ordás (2007) published considerable improvements to the transformation system for selected mature cork oak trees. Álvarez and Ordás (2007) reported an optimized protocol for *A. tumefaciens*-mediated transformation (the transformation experiments were carried out with the disarmed *Agrobacterium tumefaciens* strain AGL1harbouring the binary vector pBINUbiGUSint or pBINUbiGUSint in combination with the ternary plasmid pBBR1MCS-5.*virGN54D*) of mature embryogenic masses. Factors such as, plant genotype, explant type and time elapsed between the last subculture and inoculation, i.e. the explant pre-culture period, were found to be very important in the success of the transformation system. The interaction between inoculum density and the co-cultivation period influenced the transformation efficiency, as well. A transformation efficiency of up to 43% was obtained and it was also found that this protocol could be applied to various genotypes.

Objectives of this PhD Thesis

The aim of this thesis was to enhance the established protocol for cork oak plantlet regeneration through somatic embryogenesis. It is known that there are still some bottlenecks, especially in the control of the repetitive SE, and on maturation and germination/acclimatization stages of the SE process. Once these intermediate steps are

optimized, the rates of plant regeneration will certainly increase to levels that fulfil the needs of the cork oak breeding and industry, allowing the large-scale micropropagation of elite trees of this species. The work hereby presented intended to improve and analyse several critical steps during SE. In chapter II are described several maturation treatments performed in order to increase embryo quantity and quality and future plant conversion. Nevertheless, this process, as previously stated, is sensible to somaclonal variation. In chapter III, a genetic fidelity assessment along the process, since donor field tree through somatic embryos until regenerated plantlets is described. Chapter IV describes the importance of genotype in cell cycle variations in order to soon evaluate its potential to SE. No plant propagation genotype protocol is complete without a preservation step. In Chapter V is introduced a harmless method to cryopreserve somatic embryos with regeneration success. Chapter VI describes a ten genotype screening of selected cork-producing plants since SE until plantelet regeneration. Genetic fidelity was evaluated along the process.

These new findings open the possibility to apply SE to genotypes of interest, essential for cork oak plant breeding programs considering the production of high quality plants.

References

Álvarez R, Alonso P, Cortizo M, Celestino C, Hernández I, Toribio M, Ordás RJ (2004) Genetic transformation of selected mature cork oak (*Quercus suber* L.) trees. Plant Cell Rep 23: 218–223

Alvarez, A. and Javier, R. (2007) Improved genetic transformation protocol for cork oak (*Quercus suber* L.). Plant Cell Tiss Org Cult 91: 45-52

Alvarez, R., Alonso, P., Cortizo, M., Celestino, C., Hernández, I., Toribio, M. and Ordás, R.J. (2004) Genetic transformation of selected mature cork oak (*Quercus suber* L.) trees. Plant Cell Rep 23:218–223

Bellarosa, R. (1981) In vitro culture of *Quercus suber* L. embryos. In: Colloque International sur la Culture in vitro des essences forestiére. AFOCEL (eds.), France

Bellarosa,R.(1989) Oak (*Quercus* ssp.) In: Bajaj, Y. (eds*)* Biotechnology in Agriculture and Forestry. Vol 5. Springer Verlag. Heidelberg, pp. 387- 399

Bueno, M.A. Gómez, A., Boscaiv, M., Manzanera J.A. and Vicent, O. (1997) Stress induced haploid plant production from anther cultures of *Quercus suber*. Physiol. Plant. 99: 335-341

Bueno, M.A., Astorga, R. and Manzanera, JA (1992). Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiologia Plantarum 85, 30-34

Bueno, M.A., Gómez, A. and Manzanera, J.A. (2000) Somatic and gametic embryogenesis in *Quercus suber* L. *In*: Gupta P.K., Jain S.M., Newton R.J. (eds.). Somatic Embryogenesis in Woody Plants, Vol.6, Kluwer Academic Publishers, Netherlands, pp. 479-507

Bueno, M.A., Gomez, A., Vicente, O. and Manzanera, J.A. (1996) Stability in ploidy level during somatic embryogenesis in *Quercus canariensis*. En: Somatic cell genetics and molecular genetics of trees. Ahuja M.R., Bourjan W., Neale D. (eds) Kluwer Academic Publishers, Dordrecht. Pp. 23-28

Celestino, C., Hernández, I., Carneros, E., López-Vela, D. and Toribi,o M. (2005) La embriogénesis somática como elemento central de la biotecnología forestal. Investigación Agraria: Sistemas y Recursos Forestales. Aranda I., Cervera M.T., Pardos M. (eds).Volumen 14, pp. 345-357

Celestino, C., Hernández, I., López-Vela, D., Carneros, E., Alegre, J., Toribio, M., Fernández-Guijarro, B. and Cardo, L. (2007) First data from a field trial of *Quercus suber* plants regenerated from mature selected tress and from their half- sib progenies by somatic embryogenesis. Acta Hort. (ISHS) 748:215-218

Chalupa ,V. (1995) Somatic embryogenesis in oak (*Quercus* spp.) In: Jain S., Gupta, P. and Newton, R. eds. Somatic embryogenesis in Woody Plants.Vol 2,. Kluwer Academic Publishers, Netherlands, pp. 67-87

Deidda, P., Azzena, M. and Coinu, G. (1988) In vitro plantlet regeneration from *Quercus suber* L. Seedlings, Acta Horticulturae 227, 393- 395

Diez, J., Manjon, J.L., Dovacs, G., Celestino, C. and Toribio, M.(2000) In vitro synthesis of *Scleroderma polyrhizum* Gmel. and *Pisolithus tinctorius* ectomycorrhizae on *Quercus suber* L., plantlets raised from somatic embryos. Book of Abstracts. XIII Congress of European Mycologists. Alcala de Henares (Madrid)

Dodeman, V.L., Ducreux, G. and Kreis, M. (1997) Zygotic embryogenesis versus somatic embryogenesis (Review Article). *Jornal of Experimental Botany* 48 (313): 1493-1509

El Maataoui, M. and Espagnac, H. (1987) Neoformation de structures de type embryos somatique sur des cultures de tissue de chene liege (*Quercus suber* L.). C.R. Acad. Sc. Paris 304, (III), 83-88

El Maataoui, M., Espagnac, H. and Michaux-Ferrière, N. (1990) Histology of callogenesis and somatic embryogenesis induced in stem fragments of cork oak (*Quercus suber*) culture in vitro. Annals of Botany 66: 183- 190

El-kbiash, M.L., Ahmed, L., Abdeslam, A. and Badoc, A. (2004) Micropropagation of cork oak (*Quercus suber* L.) by axillary buds. Acta Botanica Gallica 151 (4): 401-413

Endemann, M., Hristoforoglu, K., Stauber, T. and Wilhelm, E. (2001) Assessment of age-related polyploidy in *Quercus robur* L. somatic embryos and regenerated plants using DNA flow cytometry. Biol Plant (Prague) 44: 339–345

Engelmann, F. (2004) Plant cryopreservation: Progress and prospects. In Vitro Cellular and Development Biology - Plant 40: 427- 433

Fehér, A., Pasternak, T.P. and Dudits, D. (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201–228

Féraud-Keller, C. and Espagnac, H. (1989) Conditions d'apparition d'une embryogenese somatique sur des cals issus de la culture de tissus foliares du chene vert (*Quercus ilex*). Can. J. Bot. 67:1055-1070

Fernandes, P., Rodriguez, E., Pinto, G., Roldán-Ruiz, I., De Loose, M. and Santos, C. (2008) Cryopreservation of *Quercus suber* L.somatic embryos by encapsulation-dehydratation and genetic stability evaluation. Tree Physiology 28, 1841–1850

Fernández-Guijarro, B. (1997) Embriogénesis somática en alcornoque (*Quercus suber* L.). Tesis Doctoral. E.T.S. Ingenieros de Montes. Universidad Politécnica de Madrid.

Fernandez-Guijarro, B., Celestino, C. and Toribio, M.(1996) Influence of genotype and degree of development on the induction of somatic embryogenesis in *Quercus suber* zygotic embryos. Abstract, COST 822, Workshop of WG 1, Berlin

Fernández-Guijarro, B., Celestino, C. and Toribio, M. (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell Tiss Organ Cult. 41, 99-106

Ferreira, F. (2000) The cork oak condition in Portugal. In: Oszako T, Delatour C (eds) Recent advances on oak health in Europe. Forest Research Institute, Warsaw

Gaj, M.D. (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to Arabidopsis thaliana (L.) Heynh. Plant Growth Regulat. 43:27-47

Gallego, F.J., Martínez, I., Celestino, C. and Toribio, M. (1997) Testing somaclonal variation using RAPDs in Quercus suber L. somatic embryos. Int J Plant Sci 158:563-567

Garcia- Martin, G., González- Benito, M. and Manzanera, J. A. (2001) *Quercus suber* L. somatic embryo germination and plant conversion: pretreatments and germination conditions. In Vitro Cell. Div. Biol.- Plant 37: 190- 198

García-Martín, G., Manzanera J.A. and González-Benito M.E. (2005) Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. Plant Cell Tiss Org Cult 80:171-177

Gingas, V.M. and Lineberger, R.D. (1989) Asexual embryogenesis and plant regeneration in *Quercus* . Plant Cell Tisuue and Organ Culture 17, 191-203

Goncalves, E. (2000) The Cork Report: A study on the economics of Cork. Sandy, Bedfordshire

Gonçalves, J. and Rainho, C. (1992) Micropropagação de sobreiro (*Quercus suber*) por rebentamento axilar. *In*: Actas do II Encontro sobre Montados de Sobro e Azinho, pp. 109- 118

Gonzalez-Arnao, M. T., Panta, A., Roca, W. M., Escobar, R. H.and Engelmann F. (2008) Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell, Tissue Organ Cult. 92: 1–13

González-Benito, M. E., García Martín, G. and Manzanera, J.A. (2002) Shoot development in *Quercus suber* L. somatic embryos. In Vitro Cell Dev. Biol. Plant 38: 477–480

Gonzalez-Benito, M. E.; Perez-Ruiz, C. (1992) Cryopreservation of *Quercus faginea* embryonic axes. Cryobiology 29:685-690

Gresshof, P.M. and Doy, C.H. (1972) Development and differentiation of haploid *Lycopersicon esculentum* (Tomato). Planta, 170: 161-170

Haggman, H., Jokela, A., Krajnakova, J., Kauppi, A., Niemi, K. and Aronen T. (1999) Somatic embryogenesis of scots pine: cold treatment and characteristics of explants affecting induction. Journal of Experimental Botany 50: 1769-1778

Hazarika,B.N. (2006) Morpho- physsiologycal disorders in in vitro culture of plants. Sci Hortic 108:105- 120

Hernández, I. (2007) Regeneración clonal de alcornoques adultos (*Quercus suber* L.) mediante embriogénesis somática. Tesis Doctoral. Facultad de Biologia. Universidad de Alcalá. Madrid.

Hernández, I., Celestino, C. and Toribio, M. (2003a) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Rep. 21: 759- 64

Hernández, I., Celestino, C., Alegre, J. and Toribio, M. (2003b) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. II. Plant regeneration from selected cork oak trees. Plant Cell Rep. 21: 765-70

Hernández, I., Celestino, C., Martinez, I., Hornero, J., Gallego, J. and Toribio, M. (2001) Induction of somatic embryogenesis in leaves from mature *Quercus suber* L trees. In: Riordain F (ed) Development of integrated systems for large-scale propagation of elite plants using in vitro techniques. EUR 19689 EN COST Action 822, Report of

activities 1999. Office for Official Publications of the European Communities, Luxembourg, pp 31–32

Hornero, J., Martínez I., Celestino C., Gallego, J., Torres, V. and Toribio, M. (2001) Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. Inter. J. Plant Sci. 162: 827- 833

IPCC (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 996 pp

Jiménez, V. (2005) Involvement of plant hormones and plant growth regulators on invitro somatic embryogenesis. Plant Growth Reg 47:91-110

Jörgensen, J. (1993) Embryogenesis in *Quercus petraea*. Ann. Sci. For., 50: 344-350

Kim, Y.W., Youn, Y., Noh, E.R. and Kim, J.C. (1997) Somatic embryogenesis and plant regeneration from immature embryos of five families of *Quercus acutissima.* Plant Cell Reports. 16: 869-873

Klopfenstein, N.B. and Kerl, J.G. (1995) The potential of biotechnology in temperate agroforestry practices. Agroforestry Systems 32 (1): 29- 44

Kumar, V., Naidu, M.M. and Ravishankar, G.A. (2006) Developments in coffee biotechnology—in vitro plant propagation and crop improvement. Plant Cell Tiss Organ Cult 87: 49–65

Lloyd G, McCown B (1980) Commercially- feasible micropropagation of mountain Laurel, *Kalmia latifolia* by use of shoot- tip culure. Combined Proceedings of the international Plant Propagator´s Society, 30: 421- 427

Lopes, T., Pinto, G., Loureiro, J., Costa, A. and Santos, C. (2006) Determination of genetic stability in cork oak long term somatic embryogenic cultures and derived plantlets using microsatellite markers. Tree Physiology 26:1145- 1152

Loureiro, J., Pinto, G., Lopes, T., Dolezel, J. and Santos C (2005) Assessement of ploidy stability of the somatic process in *Quercus suber* L. Using flow citometry. Planta 221: 815-822

Manjon, J.L., Diez, J., Celestino, C. and Toribio, M. (1998) Mycorrhization of vitroplants raised from somatic embryos of cork oak (*Quercus suber* L.). The establishment of the beneficial rhizosphere. Book of Abstracts. Inter-COST meeting of 821, 822, 830 and 831, Edinburgh, UK

Manzanera J.A. and Pardos, J.A. (1990) Micropropagation of juvenile and adult *Quercus suber* L. Plant Cell Tissue and Organ Culture 21: 1-8

Manzanera, J.A., Astorga, R. and Bueno, M.A. (1993). Somatic embryo induction and germination in *Quercus suber* L. Silv. Genet. 42, 90-93

Manzanera, J.A., Bueno, M.A. and Pardos, J.A. (1996) *Quercus robur* L. (pedunculate oak). En: Biotechnology in agriculture and forestry. Bajaj Y.P.S. (ed) Springer, Berlin Heidelberg New York. Vol. 35, pp. 321-341

Mauri, P.V. and Manzanera, J.A. (2003) Induction, development and maturation of holm oak (Quercus ilex L.) somatic embryos. Plant Cell Tiss. Org. Cult., 74:229-235

Merkle, A. and Nairn, J.C. (2005) Hardwood tree biotechnology. In Vitro Cell DevBiol Plant 41:602-619

Merkle, S.A., Bailey, R.L., Pauley, B.A., Neu, K.A., Kim, M.K., Rugh, C.L. and Montello, P.M. (1997) Somatic embryogenesis from tissues of mature sweetgum trees. Can. J. For. Res. 27: 959-964

Merkle, S.A., Parrott, W.A. and Flinn, B.S. (1995). Morphogenic Aspects of Somatic Embryogenesis. In: Thorpe, A. T. (eds.) *In vitro* embryogenesis in plants. Kluwer Academic publisher. Dordrecht, pp. 155- 204

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497

Natividade, J.V. (1990) Subericultura. Ministério da Agricultura, Pescas e alimentação. Direcção- Geral das florestas.Lisboa (2ª edição), 387 pp

Ostrolucka, M.G. and Petrova, A. (1991) The occurrence of somatic embriogénesis in the species *Quercus cerris* L. Biologia, 46: 9-14

Park, Y-S. (2002) Implementation of conifer somatic embyogenesis in clonal florestry: technical requirements and deployment considerations. Ann For Sci 59: 651-656

Park, Y-S., Barrett, J.D. and Bonga, J.M. (1998a) Appications of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol Plant 34:231-239

Pinto G, Valentim H, Costa A. Santos C (2002) Somatic embryogenesis in leaf plant calluses of mature *Quercus suber*. In Vitro Cell. Div. Biol.- Plant 38: 569- 572

Pinto, G. (2002) Embriogénese somática e conversão de embriões em plantas de *Quercus suber* L. Master thesis. Trás- os- Montes e Alto Douro. Portugal.

Pinto, G., Park, Y-S., Neves, L., Araujo, C. and Santos, C. (2008) Genetic control of somatic embryogenesis in *Eucalyptus globulus* Labill. Plant Cell Reports 27: 1093- 1101

Pinto, G., Park, Y-S., Silva, S., Araújo, C., Neves, L. and Santos C (2008b) Factors affecting maintenance, proliferation, and germination of secondary somatic embryos of *Eucalyptus globulus* Labill. Plant Cell Tissue and Organ Culture 95: 69-78

Pintos, B., Bueno M.A, Cuenca, B. and Manzanera, J.A (2008) Synthetic seed production from encapsulated somatic embryos of cork oak (*Quercus suber* L.) and automated growth monitoring. Plant Cell Tiss Organ Cult 95:217–225

Puigderrajols, P., Fernández- Guijarro, B., Toribio, M. and Molinas, M. (1996) Origin and early development of secondary embryos in *Quercus suber* L. Int. J. Plant Sci. 157 (6), 674-684

Puigderrajols, P., Mir, G. and Molinas, M. (2001) Ultrastructure of early secundary embryogenesis by multicelluar and unicellular pathways in cork oak (*Quercus suber* L.) Annals of botany 87: 179-189

Quercus suber L. En: Pardos J.A., Ahuja M.R., Elena-Rosello R. (eds.) Biotechnology of Trees. Investigación Agraria, Sistemas y Recursos Forestales, fuera de serie nº 4. INIA, Madrid. Pp. 105-110

Rancillac, M., Klinger, A., Klinger, S. and Millet, B. (1996) Preliminary investigations on somatic embryogenesis from leaf discs of red oak (*Quercus rubra* L.*).* Plant Growth Regulation. 20, 67-73

Romano, A. and Martins- Loução, M.A. (1992) Micropropagation of mature cork-oak (*Quercus suber* L.): establishment problems. Scientia genmdensis 18: 17-27

Romano, A. and Martins- Loução, M.A. (1999) In vitro cold storage of cork oak shoot cultures. Plant Cell Tiss Organ Cult 59: 155- 157

Romano, A. and Martins- Loução, M.A. (2003a) Strategies to improve rooting and acclimatization of cork oak. Acta Hort. (ISHS) 616: 275-278

Romano, A. and Martins- Loução, M.A. (2003b) Water loss and morphological modifications in leaves during acclimatization of cork oak micropropagated plantlets . Acta Hort. (ISHS) 616: 439-442

Romano, A., Noronha, C. and Martins-Loução, M.A. (1995) Role of carbohydrates in micropropagation of cork oak. Plant Cell Tiss Organ Cult 40(2): 159-167

Sakai, A. (2004) Plant cryopreservation. In: Fuller B. J. Lane N. Benson E. E. (eds) Life in the frozen state. CRC, Boca Raton, pp 329–345

Sánchez N, Manzanera J, Pintos B, Bueno M (2005) Agrobacterium-mediated transformation of cork oak (*Quercus suber* L.) somatic embryos . New Forest 29: 169-176

Santos, C., Loureiro, J., Lopes, T. and Pinto, G. (2007) Genetic fidelity analyses of in vitro propagated cork oak (*Quercus suber* L.) In: Protocols for micropropagation of woody

and fruit trees. Mohan Jain S., Häggman H. (eds) Springer. Dordrecht, The Netherlands. 500pp 1st. ed

Sasamoto, H.and Hosoi, Y. (1989) Somatic embryogenesis in suspension cultures of Quercus serrata Thunb. J. Jpn. For. Soc., 71:20-22

Schenk, R.U. and Hildebrandt, A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Srivastava, P.S. and Steinhauer, A. (1982) In vitro culture of embryo segments of *Quercus lebani*: organogenesis and callus growth as a differential response to experimental conditions. Z. Pflanzenphysiol., 106:93-96

Toribio, M. (1986) Callus initiation and primary morphogenic responses from *Quercus suber* L. cotyledons cultured in vitro. Proceedings of the 18th International Union of Forest Research Organization World Congress, Div. 2, Ljubljana, Yugoslavia. Publications of the Yugoslav IUFRO World Congress Organizing Committee, 2:863

Toribio, M., Celestino, C. and Molinas, M. (2005) Cork oak, *Quercus suber* L. In: Protocol for somatic embryogenesis in woody plants. Jain S.M., Gupta P.K. (eds) Forestry Sciences. Vol. 77, pp. 445-458

Toribio, M., Celestino, C., Gallego, J. and Martínez , I. (2000) Induction of somatic embryogenesis in tisues from mature oak trees. In: Ó Rídordáin F. (ed.) Development of integrated systems for large- scale propagation of elite plants using in vitro techniques. Reports of activities, 1998. EUR- COST Action 822. Luxembourg, pp. 236-237

Toribio, M., Celestino, C., Gallego J. and Martínez I. (1998) Induction of somatic embryogenesis in tissues from mature oak trees. En: Development of integrated systemsfor large-scale propagation of elite plants using in vitro techniques. Ríordáin F.Ó. (ed).

8. Bibliografía- 180 -EUR 19237 – COST Action 822. Office for Official Publications of the European Communities, Luxembourg. pp. 236-237

Toribio, M., Fernández, C., Celestino, C., Martínez, M.T., San-José, M.C. and Vieitez A.M. (2004) Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell, Tissue Org. Cult. 76: 283-287

Valladares, S., Toribio, M., Celestino, C. and Vieitez, A.M. (2004) Cryopreservation of embryogenic cultures from mature *Quercus suber* trees using vitrification. CryoLetters 25: 177-186

Vendrame, W.A., Holliday, C.P., Montello, P.M., Smith, D.R. and Merkle, S.A.(2001) Cryopreservation of yellow-poplar and sweetgum embryogenic cultures. New Forests 21 (3): 283-292

Vogiatzakis, I.N. and Careddu, M.B. (2003) Mapping the distribution and extent of *Quercus suber* habitats in Sardinia: a literature review and a proposed methodology .Geographical Paper No. 171. Series Editor: A M Mannion

Von Arnold, S., Sabala, I., Bozhkovi, P., Dyachok, J. and Filanova, L. (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Organ Cult 69: 233-249

Wilhelm, E. (2000) Somatic embryogenesis in oak (*Quercus* spp.) In vitro Cellular and Development Biology- plant 9: 349-357

Wilhelm, E., Endemann, M., Hristoforoglu, K., Prewein, C. and Tutkova, M. (1999) Somatic embryogenesis in oak (*Quercus robur* L.) and production of artificial seeds. In: Proceedings of application of biotechnology to forest genetics. Biofor 99, Vitoria-Gasteiz

Chapter II

Enhancement of maturation and germination of somatic embryos of cork oak

Chapter section submitted as original article in SCI journal:

Fernandes P, Silva S, Canhoto J, De Loose M, Santos C (2011) Enhancement of maturation and germination of somatic embryos of cork oak (*Quercus suber* L.) *Trees- Structure and Function* (submitted)

Abstract

Despite cork oak (*Quercus suber* L.) somatic embryogenesis (SE) has been achieved from adult trees, for this process to be useful in large scale cloning, the maturation step still needs to be better understood. The effects of different light and temperature conditions, abscisic acid (ABA), polyethylene glycol (PEG) and sucrose were evaluated on somatic embryo maturation. ABA or sucrose only stimulated maturation when applied together with chilling/dark conditions. Addition of ABA in dark conditions promoted cellular organization, as well as storage compound accumulation, mostly starch. Chilling alone was ineffective to increase cell mass and, at dark, promoted little or no differentiation, resulting in immature structures. The highest mass increase and number of matured embryos was obtained with MSWH medium supplemented with 10% PEG at 24±2 ºC and 99±5 $\mu mol.m^{-2}.s^{-1}$ photon flux. This condition also promoted starch reserves and phenolic compounds accumulation in a similar pattern to the one found in zygotic embryos. The best rate of conversion, 20% of plants converted, was achieved when mature embryos were transferred back to basal MS medium and submitted to a 4ºC treatment for two months. These data are a significant advance in improving and manipulating both cork oak SE maturation and germination stages, crucial for further industrial applications.

Keywords: ABA, cork oak, histocytology, light, maturation, PEG, temperature, somatic embryogenesis.

Introduction

Cork oak (*Quercus suber* L.) plays an important role in Southern European and Northern African ecosystems and is the base for the cork transformation industry. Although it is a protected species in Portugal, cork oak received a threatened status (FAO, 1996) and national strategies are being developed for efficiently regenerating selected genotypes. Somatic embryogenesis (SE) is frequently regarded as the best system for propagation of superior genotypes (Park, 2002), mostly because both root and shoot meristems are present simultaneously allowing a high output of plant conversion. *Q. suber* SE is currently achieved through leaf explants of mature plants (Hernandez *et al.*, 2003a; Lopes *et al.*, 2006; Pinto *et al.*, 2002; Toribio *et al.*, 1998). However, these somatic embryos often show low conversion frequencies, jeopardizing the use of SE as an effective method to scale up plant propagation.

Some authors have pointed out the deficient maturation during SE as the main cause of these drawbacks (Chalupa, 2005; Hernandez *et al.*, 2003a; Hernandez *et al.*, 2003b). An adequate nutrient supply to the somatic embryos to further support their germination remains the main bottleneck to efficient conversion of cork oak somatic embryos (Hernandez *et al.*, 2003a; Hernandez *et al.*, 2003b). Several strategies have been pursued to better achieve somatic embryo maturation. Fernández-Guijarro *et al.* (1995) reported that low availability of total nitrogen and high concentrations of reduced nitrogen increased embryo proliferation, morphogenesis accuracy and synchronization in cork oak embryos. Other approaches have highlighted the role of abscisic acid (ABA) to improve somatic embryos maturation (Kermode, 1995). This plant hormone promotes reserve accumulation in e.g. *Hevea brasiliensis* (Cailloux *et al.*, 1996) and *Pinus elliottii* (Liao and Amerson, 1995) and reduces the frequency of abnormal phenotypes in *Hevea brasiliensis* somatic embryos (Etienne *et al.*, 1993) and in secondary embryos of *Betula pendula* (Nuutila *et al.*, 1991). In cork oak, Garcia-Martin *et al.* (2001) reported that exogenous ABA prevented these unwanted side-effects during the early SE stages.

Osmotic stress is also an important factor for in vivo and in vitro embryo development and maturation (Korbes and Droste, 2005; Shoji *et al.*, 2006). Application of polyethylene glycol (PEG) has become a routine method for stimulating embryo maturation (Rai *et al.*, 2009). PEG mimics the naturally occurring water stress on seeds during late stages of maturation (Stasolla *et al.*, 2003). Also, PEG has been shown to increase deposition of storage proteins similar in abundance and electrophoretic mobility to those accumulated in zygotic embryos (Misra *et al.*, 1993).

Exposure of somatic embryos to cold also improved germination in *Fraxinus excelsior*, (Capuana *et al.*, 2007) and *Picea glauca* (Pond, 2005). (Fernández-Guijarro *et al.*, 1995) showed that the rates of germination increased when somatic embryos obtained from cork oak young seedlings were first kept under light conditions for maturation and followed by dark storage at 4 °C. Usually, absence or low light photon fluxes during maturation increases the index of final conversion (Vila *et al.*, 2007). For example, darkness favoured *Q. robur* acorns (Finchsavage and Clay, 1994) and *Picea* sp. somatic embryo germination (Attree *et al.*, 1995).

Carbohydrate source may also influence somatic embryo yield and improve somatic embryos morphology (Vila *et al.*, 2007). Garcia-Martin *et al.* (2001) reported that germination of cork oak somatic embryos required the supply of a carbon source. Also sucrose concentration affected germination of somatic embryos in *Quercus ilex* L. and *Psidium guajava* L. (Mauri and Manzanera, 2004; Rai *et al.*, 2008).

Despite the descriptions of external stimuli influencing somatic embryos maturation and conversion, their influence on the accumulation of storage compounds (starch, proteins or lipids) and further correlation with embryo quality are rarely documented in dicotyledonous woody species (Robichaud *et al.*, 2004). The histological descriptions made so far for *Quercus* sp. somatic embryogenesis system have been focused on the early stages of induction (Elmaataoui *et al.*, 1990) and on early steps of secondary somatic embryos origin (Puigderrajols *et al.*, 2001), while no studies were performed to clarify reserve accumulation.

Here we hypothesized that changing environmental signalling will change somatic embryos reserve accumulation and this will improve somatic embryo maturation and germination. We studied the effects of ABA, stratification, carbohydrate availability, culture medium osmotic alteration, light and temperature effects in cork oak somatic embryos maturation. Also, we correlate the effects of the best conditions for promoting somatic embryo maturation with acorns reserves accumulation profile.

Materials and Methods

Plant material

Quercus suber leaf samples were obtained during the months of May and June from a 60 year-old cork oak tree (Águeda, Portugal). Somatic embryogenesis was induced according to (Pinto *et al.*, 2002). Briefly, leaves were washed in 0.1% (w/v) Benlate (50% benomyl; Rhône Poulenc Agro, Du Pont de Nemours Inc. Wisconsin, USA) and in distilled sterile water. For embryogenic callus induction, explants were placed in Petri dishes on MS (Murashige and

Skoog, 1962) medium with 30 g/L sucrose, 3 g/L gelrite, pH adjusted to 5.8 and supplemented with 1 mg/L 2,4–dichlorophenoxyacetic acid (2,4-D) and 2 mg/L zeatin. Cultures were maintained at 24±1ºC in the dark. After 3 weeks, cultures were transferred to a photoperiod of 16 h and a photon flux of 98±2 µmol.m^{-2}.s^{-1}. As soon as the first somatic embryos appeared they were isolated and transferred to Petri dishes containing fresh MS medium without growth regulators supplemented with 30 g/L sucrose, 3 g/L gelrite, pH adjusted to 5.8 (MSWH). Repetitive somatic embryogenesis was maintained in MSWH and transferred monthly to fresh culture medium. Cryopreservation is currently used to avoid culture aging and inherent mutations following the protocol developed by (Fernandes *et al.*, 2008).

Maturation treatments

Clusters of secondary somatic embryos containing approximately 10 embryos at the globular stage were isolated and placed in solid (3 g/L gelrite) MSWH media supplemented with PEG 6000 (Merck): 1, 5 and 10%; ABA (Sigma): 1, 10 and 25 mg.L^{-1}; Sucrose (Duchefa): 15 and 60 g.L^{-1}. All treatments were combined with a photoperiod of 12h of a photon flux of 99±5 µmol.m^{-2}.s^{-1} versus dark and chilling (4±2 ºC) versus normal temperature conditions (24±2 ºC). During two months both fresh weight (FW) and number of cotyledonary somatic embryos were evaluated. Media was replaced every four weeks.

Histochemical analysis of storage compounds

For cyto-histochemical characterization and reserves analyses, samples were collected from the following material: a) cotyledons of zygotic embryos from mature acorns and b) secondary somatic cotyledonary embryos after two months of maturation in each previously referred condition. Samples were fixed in 2.5% glutaraldehyde in 1.25% (w/v) piperazine-N,N'-bis-(2-ethanesulfonic acid) (Pipes) buffer (pH 7.4) for 3 h and washed in Pipes. Tissues were post-fixed in 1% (w/v) osmium tetroxide in Pipes buffer for 1h, rinsed in the same buffer and dehydrated through a graded ethanol series and embedded in a graded low-viscosity epoxy resin (Epon, EMS). The blocks were polymerised at 60ºC for 48 h. Ultra-thin sections were cut with a LKB ultra-microtome using a diamond knife and collected on uncoated copper grids. The sections were stained with uranyl acetate for 15 min and lead citrate for 10 min (Reynolds 1963) and observed with a Siemens Elmiskop-101 transmission electron microscope at 80 kV.

For reserve accumulation analyses, semi-thin sections (1-2 μM) from the material embedded for electron microscopy were used. Sections were then stained with Sudan Black B (0.3%) for lipidic reserves analyses, with Coomassie Brilliant Blue (0.25%) for protein accumulation analyses, or by Periodic Acid-Schiff reaction (PAS) for carbohydrate analyses. Samples were analysed in a Nikon Eclipse 80i light microscope and digital photographs were taken using a Leica DC 200 digital camera. For quantitative analyses (starch and phenolic compounds), 40 cells sections from both periphery and cortical areas (20 each) within three semi-thin cross sections from different somatic embryos were randomly screened for each condition.

Conversion treatments

Based on maturation results, particularly reserve substance accumulation, weight increase and cotyledonary evolution, somatic embryos which have matured in 10% (w/v) PEG-supplemented culture medium, were selected for plant conversion studies. Somatic embryos that matured spontaneously, 10–15 mm length with an average fresh weight of 150 mg and without signs of secondary somatic embryogenesis (SSE) were isolated and placed on MS culture medium. The role of ABA (10 mg. L^{-1}), PEG (10%), stratification and luminosity on somatic embryo conversion was evaluated.

Statistical analyses

For each parameter, values are given as Mean ± Standard Error as calculated from data from four independent experiences (with five replicates in each experiment). The mean separations were carried out using Duncan's multiple range tests (Duncan, 1955) and significance was determined at $P < 0.05$ using ANOVA. All statistical analysis was performed using the software package SPSS for *Windows* (version 17.0.0, SPSS Inc.)

Results

Maturation

To evaluate SE maturation, zygotic embryos were analysed for comparative purposes with somatic ones. Zygotic embryos showed a defined protoderm surrounding well organized regions and procambium cells. Cells were rich in starch and polyphenolics (Figure 1a, b), while no protein bodies were observed. Ultrastructural analyses show starch and lipid reserves allocated in typically poorly differentiated cells (Figure 1g).

PEG effect

The three tested PEG concentrations increased the mass growth and the number of cotyledonary-staged embryos in relation to the control ($P<0.05$). The application of 10% (w/v) PEG 6000 at light and 24ºC resulted in nearly ten times more cotyledonar embryos and more than a four times mass increase in respect to control (Table 1). Contrarily, PEG application in dark conditions did not affect weight variation or the evolution to the cotyledonary stage, comparatively to control (Table 1). Under chilling conditions, no significant variations were visible ($P<0.05$; Table 1). Somatic embryos exposed to PEG conditions (mainly 10% PEG) accumulated large amounts of storage substances, showing particularly a homogeneously distribution of starch, a profile similar that of zygotic embryos (Figure 1d, e). Starch grain per cell section counting revealed that any of the PEG concentrations assayed lead to an increase of starch content at the peripheral areas, though, in light conditions at 24ºC, 10% PEG showed values identical to zygotic embryos and much higher than for control (Figure 2). On the other hand, under dark or chilling treatments no changes were observed and accumulation of starch grains per cell section was always lower than in the controls.

Table 1: Influence of different PEG concentrations together with different maturation conditions on oak somatic embryos maturation, during two months. For the same column, different letters mean significantly different means ($p<0.05$).

Luminosity ($\mu mol.m^{-2}.s^{-1}$)	**Temperature (ºC)**	**PEG 6000 (%)**	**Weight increase (%)**	**Number of cotyledonar embryos**
99±5[a]	24±2	0	588,7 **abcd**	55 **abc**
99±5	24±2	1	1014,4 **cd**	258 **e**
99±5	24±2	5	1135,0 **d**	411 **f**
99±5	24±2	10	2284,8 **e**	484 **f**
Dark	24±2	1	830,8 **abcd**	40 **ab**
Dark	24±2	5	714,7 **abcd**	8 **a**
Dark	24±2	10	553,5 **abcd**	67 **abc**
Dark	4±2	1	9,8 **a**	9 **a**
Dark	4±2	5	31,1 **a**	15 **a**
Dark	4±2	10	23,8 **a**	13 **a**

[a] Control experiment.

Phenolic compounds were abundant in zygotic embryos (Figure 1a; Figure 3) being also identified only in somatic embryos derived from 10% PEG, light and 24ºC conditions (Figure 1d; Figure 3). Despite that these values were twice as high as those obtained for control

(Figure 3), they constituted half of that verified for zygotic embryos. Also under these conditions, a clear cellular organization was visible, and occasional xylogenesis was observed (Figure 1c). Moreover, at the ultrastructural level is possible to identify the formation of plastids and several mitochondria, as well as some reserve substances accumulation (Figure 1i).

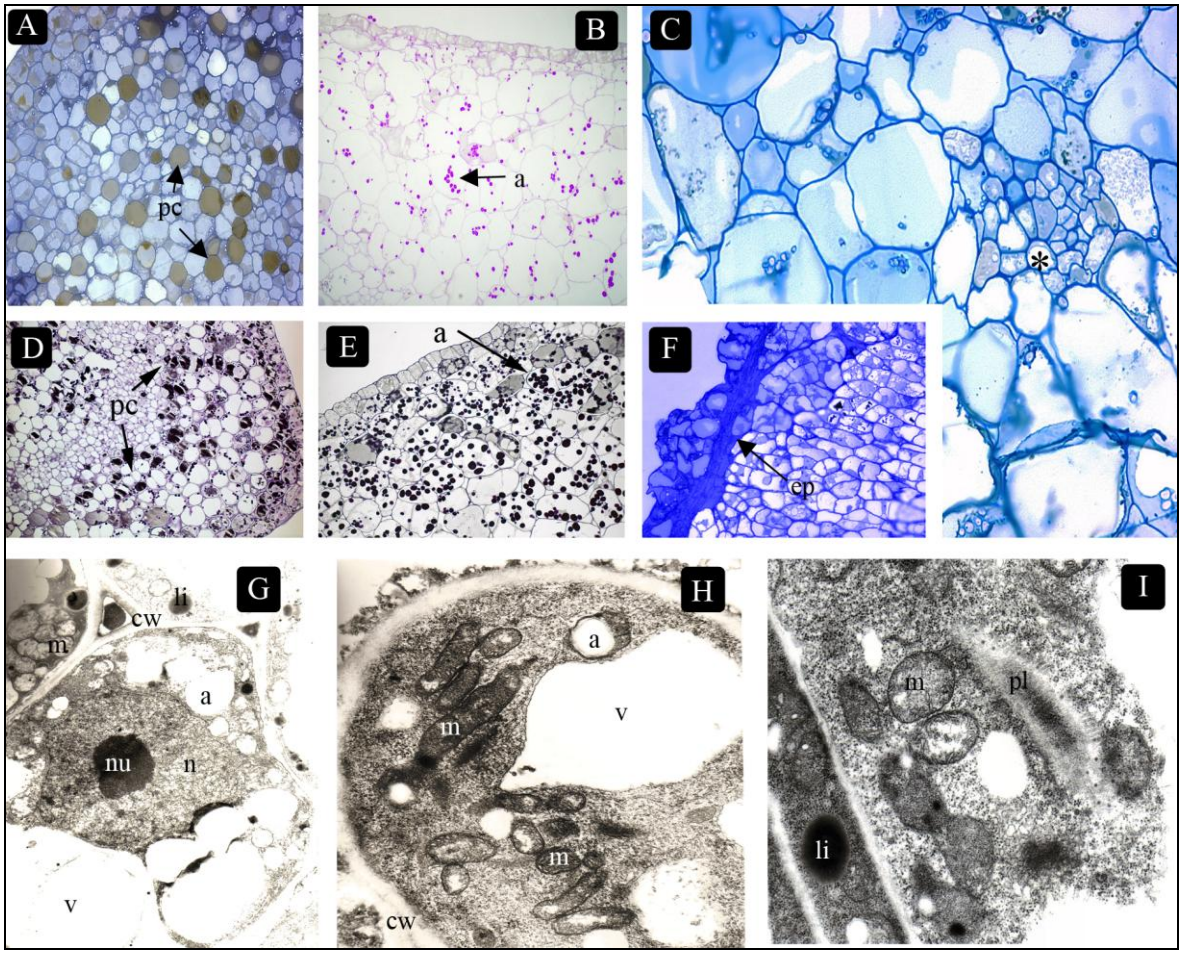

Figure 1: Histological and ultrastructural analysis. A, B: sections of a zygotic embryo stained with Coomassie Brilliant Blue and PAS, respectively. C: Section of somatic embryos cultured on PEG 10% media, light and 24 ºC, stained with Coomassie Brilliant Blue. D, E: Section of somatic embryos cultured on PEG 10% media, light and 24 ºC, stained with PAS. F: Section of a somatic embryo maturated in ABA 10 mg.L^{-1}, dark and chilling conditions, stained with Coomassie Brilliant Blue. G: Ultrastructural section detail of a mature zygotic embryo cell showing the organizational level. H: Ultrastructural detail of a somatic cotyledonar embryo cell cultured in sucrose 60 g.L^{-1} supplemented media, dark and 24 ºC. I: Ultrastructural detail of a somatic embryo cell cultured with PEG 10% in the dark at 24 ºC, presenting plastid membrane formation and a visible cytoskeleton. a: starch; li: lipids; m: mitochondria; cw: cell wall; nu: nucleolus; n: nucleus; v: vacuole; pc: phenolic compound; pl: plastid; *: vascular cells; ep: epidermis. Bars represent 0.05 mm for light microscopy images and 1 µm for TEM images.

ABA effect

ABA addition did not influence weight increase (P>0.05), reserve substances accumulation or somatic embryo transition to the cotyledonary stage (Table 2). Under dark conditions, all tested ABA concentrations (1, 5 and 10 mg.L^{-1}) promoted the differentiation of cotyledonary-staged embryos (Table 2). Moreover, a trend for starch accumulation was observed under these conditions though always lower than those of zygotic embryo values (Figure 2). ABA-treated embryos exposed to light were completely unorganized, characterized by the absence of protoderm and procambium, while in dark conditions the differentiation of a sub-protoderm zone composed of several cell layers could be observed (Figure 1f).

Phenolic accumulation was only detected under dark conditions, but mean values for these compounds were lower than in control and highly heterogeneous among samples (Figure 3).

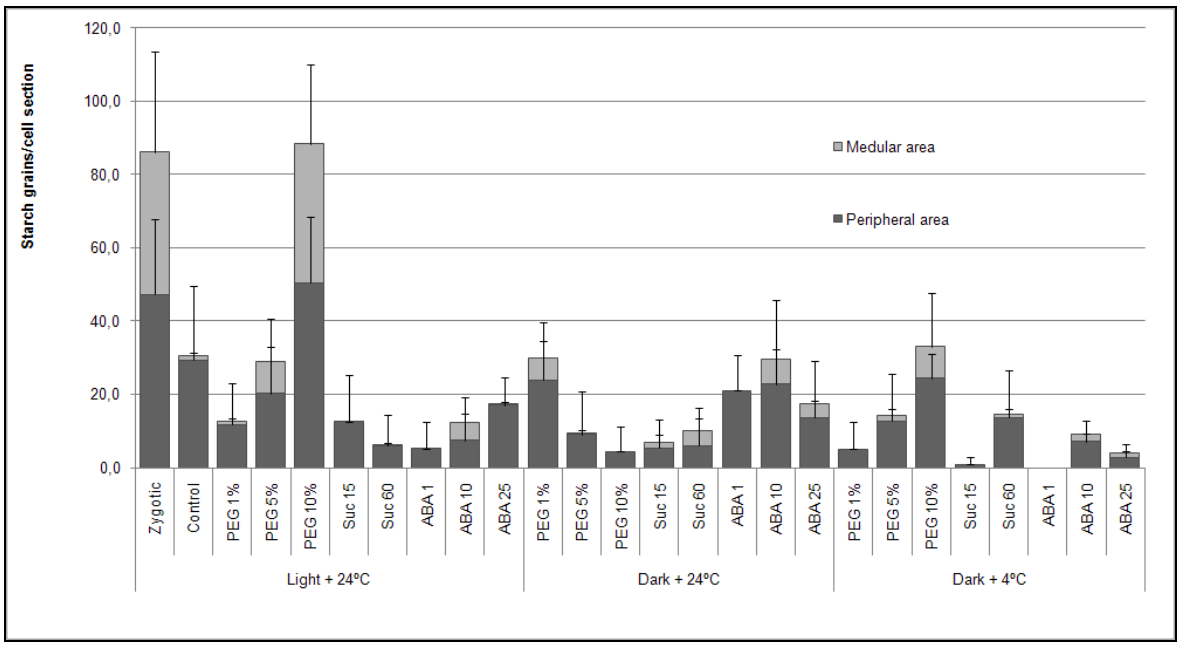

Figure 2: Starch grains counting per cell section of microscopic slides preparation. Two areas were considered: medullar and peripheral area. Vertical bars represent standard deviation.

Sucrose effect

The variation of sucrose availability in the culture medium, comparatively to the control, resulted in small differences. No significant stimulus of mass volume was found within somatic embryos exposed to different sucrose levels. Concerning the number of cotyledonary somatic embryos, and relatively to control, when sucrose availability was reduced, this number increased two and three times, in both dark and light conditions, respectively.

When starch accumulation was the parameter analysed no significant differences were obtained. It was also found that somatic embryo cells grown in 60 g sucrose supplemented media were highly vacuolated possessing several mitochondria and some plastids with starch (Figure 1h).

Table 2: Influence of different ABA concentrations together with different maturation conditions on oak somatic embryos maturation, during two months. For the same column, different letters mean significantly different means ($p<0.05$).

Luminosity ($\mu mol.m^{-2}.s^{-1}$)	Temperature (ºC)	ABA ($mg.L^{-1}$)	Weight increase (%)	Number of cotyledonar embryos
99±5[a]	24±2	0	588.7 **abcd**	55 **abc**
99±5	24±2	1	847.1 **abcd**	25 **ab**
99±5	24±2	10	1125.4 **cd**	56 **abc**
99±5	24±2	25	1118.6 **cd**	34 **ab**
Dark	24±2	1	539.7 **abcd**	142 **cd**
Dark	24±2	10	696.6 **abcd**	143 **cd**
Dark	24±2	25	688.3**abcd**	84 **abcd**
Dark	4±2	1	31.7 **a**	3 **a**
Dark	4±2	10	36.0 **a**	6 **a**
Dark	4±2	25	49.3 **a**	2 **a**

[a] Control experiment.

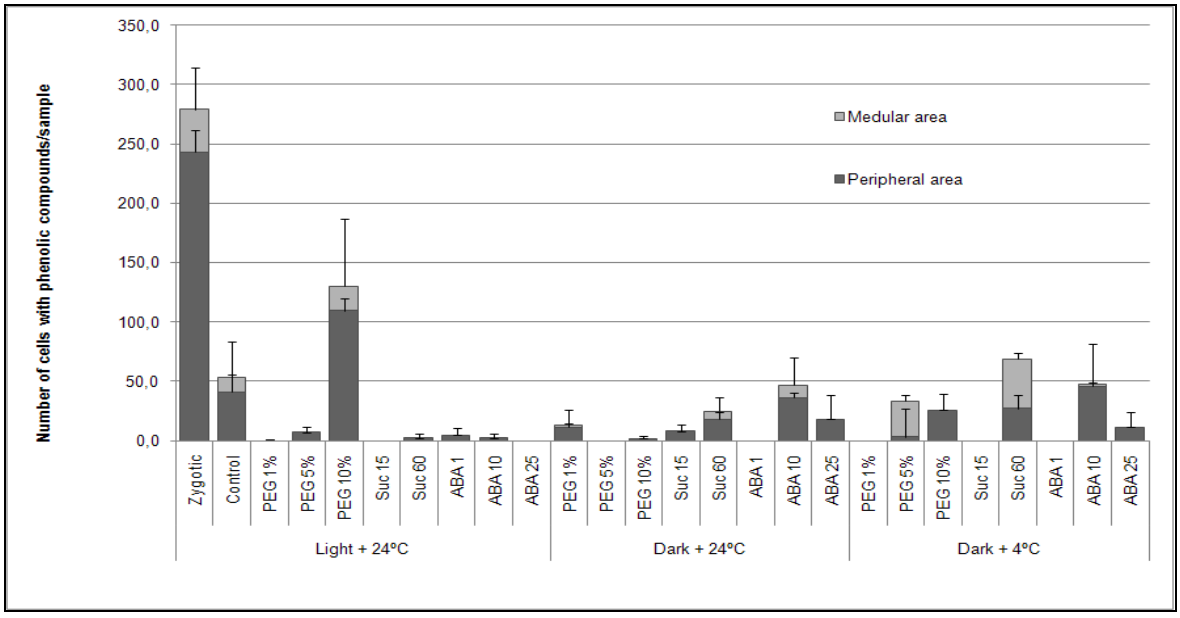

Figure 3: Number of cell sections containing polyphenolics. Two areas were considered: medullar and peripheral area. Vertical bars represent standard deviation.

Table 3: Influence of different sucrose concentrations together with different maturation conditions on oak somatic embryos maturation, during two months. For the same column, different letters mean significantly different means ($p<0.05$).

Luminosity ($\mu mol.m^{-2}.s^{-1}$)	Temperature (ºC)	Sucrose ($g.L^{-1}$)	Weight increase (%)	Number of cotyledonar embryos
99±5[a]	24±2	30	588.7 **abcd**	55 **abc**
99±5	24±2	15	930.3 **bcd**	110 **bcd**
99±5	24±2	60	939.7 **cd**	69 **abc**
Dark	24±2	15	804.9 **abcd**	167 **d**
Dark	24±2	60	292.7 **abc**	32 **ab**
Dark	4±2	15	86.1 **a**	57 **abc**
Dark	4±2	60	109.4 **ab**	58 **abc**

[a] Control experiment.

Conversion

After selection of maturated embryos in 10% (w/v) PEG supplemented culture medium, the effect of several conversion conditions was evaluated. Conversion rates were higher when matured embryos were transferred to basal medium and submitted to cold and dark during two months, resulting in 20% conversion to emblings (Table 4). These conditions also promoted elongation of both apical and root meristems 45% and 40%, respectively, despite only 20% synchronization was achieved. Chilling at 4ºC was crucial for shoot apical meristem (SAM) elongation. Even though root apical meristems (RAM) appeared at 24ºC, no SAM elongated, resulting in no conversion. Moreover, for all treatments, secondary embryogenesis was always observed. In this case PEG and ABA seem to have a positive effect in reducing SSE when cultures were at 24º C under light. This same effect was achieved when cultures were chilled in the dark. However, PEG and ABA only enhanced SAM and RAM emergence without any synchronization at all.

Discussion

It is well known that external stimuli influence somatic embryo maturation (Gaj, 2004). Based on this, we demonstrate here that, for cork oak, the use of ABA, osmotic agents (PEG) or different concentrations of sucrose are essential, *per se* or together with other parameters (e.g. luminosity and/or temperature) for efficient somatic embryo maturation.

Table 4: Influence of different conversion conditions on oak somatic embryos after two months. SAM: shoot; RAM: root; ESS: secondary somatic embryogenesis. For the same column, different letters mean significantly different means ($p<0.05$).

Conversion treatment	SAM (%)	RAM (%)	Conversion (%)	SSE (%)
MSSH + 24ºC + Light[a]	0	20	0	80 **e**
MSSH + PEG (10%) + 24ºC + Light	0	5	0	35 **abc**
MSSH + ABA (10 mg.L^{-1}) + 24ºC + Light	0	0	0	30 **abc**
MSSH + 4ºC + Dark	45 **a**	40 **a**	20 **a**	75 **e**
MSSH + PEG (10%) + 4ºC + Dark	20 **b**	10 **b**	0	65 **d**
MSSH + ABA (10 mg.L^{-1}) + 4ºC + Dark	5 **c**	5 **c**	0	20 **abc**

[a] Control experiment.

Zygotic embryos showed metabolically active cells, also rich in starch and phenols, but with no detectable storage protein vacuoles. Tereso *et al.* (2007) describe for *Pinus pinaster* that protein bodies accumulation depends on the embryo stage: in early maturation stages, protein bodies are scarce, while in advanced phases their presence is abundant, reinforcing that not detecting protein bodies is not indicative of their absolute absence, but probably indicates a low protein-presence stage.

Starch and phenolic compounds were also abundant in cork oak both zygotic and somatic embryos, depending on the maturation conditions. This is also very common during *in vitro* cultures, particularly in woody species (Ofei-Manu *et al.*, 2001; Pinto *et al.*, 2010). Although some studies indicate that phenolic composites inhibit morphogenic processes (Ozyigit *et al.*, 2007), recent cases report that somatic embryos are produced from cells/tissues that accumulate phenols or are situated in the neighbourhood of phenol-rich cells which seem to have a role on somatic embryo isolation from the mother tissues (Reis *et al.*, 2008a). Nevertheless, diferent treatment conditions resulted in diferent maturation enhancements, which will be discussed further.

PEG effect

From all the conditions tested, PEG plus light gave the best results for stimulating both somatic embryogenic development and maturation, promoting both mass increase rate and number of cotyledonary embryos. The importance of the osmotic potential in SE has been widely referred (Attree *et al.*, 1991; Attree *et al.*, 1995; Pullman and Johnson, 2009; Tereso *et al.*, 2007).

Maturation in PEG-supplemented medium showed a histological somatic embryo development similar to the zygotic one. Starch and phenolic compounds were found in the same proportions and distribution was identical. Despite number of starch grains and starch grain size can change with embryo maturity in such a way that the same number of starch grains could indicate much more starch (Sánchez-Romero *et al.*, 2006), we observed similarity between size and distribution. Moreover, protein accumulation was not detectable. Shoemaker *et al.* (1987) showed, for cotton somatic embryos, that protein accumulation fluctuates throughout SE ontogeny: the higher levels occur during the globular and heart-shaped stages. Moreover, protein precursors (60-70 kD) had been found during these phases. This strengthens the idea that storage proteins are not always present during all SE process and only metabolic proteins may be present (Cangahuala-Inocente *et al.*, 2009).

PEG-treated somatic embryos showed a similar pattern to acorns of both starch accumulation and phenolic compounds' distribution, presenting organized structures: the presence of vascularised structures is abundant. In these somatic embryos, the protoderm was usually well defined and lipid-rich. Using white spruce somatic embryos, Stasolla *et al.* (2003) supported that PEG was essential to attain greater number and high quality somatic embryos. They report that PEG application increased the number of transcribed genes during the maturation phase, improving somatic embryo quality. Linossier *et al.* (1997) shown that the application of PEG in *Hevea brasiliensis* somatic embryos resulted in a fast maturation and embryogenic development, and increased the formation of new globular embryos. Similar results were obtained for *Abies cephalonica* somatic embryos by Krajnakova *et al.* (2009). Moreover, PEG application is usually reported as an inductive agent of protein and starch accumulation (Robichaud *et al.*, 2004; Tereso *et al.*, 2007). Attree *et al.* (1991) observed a 3-fold increase in the maturation frequency of white spruce embryos after application of PEG. Such embryos closely resemble their zygotic counterparts in low moisture level and ability to tolerate desiccation (Attree *et al.*, 1995).

Other important aspect to discuss is that PEG only presented a stimulatory effect in conjunction with light. In conifers, PEG is usually applied in culture media to stimulate somatic embryo maturation under dark conditions. However, PEG did not stimulate SE maturation without light in American chestnut (Robichaud *et al.*, 2004) and Brazilian grape tree (Motoike *et al.*, 2007). All in all, despite a clear interaction between PEG and light, further studies are needed to disclose the mechanisms of this process.

ABA effect

ABA interferes with reserve accumulation and somatic embryo transition to the cotyledonary stage and, in general, dark apparently exerted a stimulatory effect. Karkonen (2000) suggested that ABA is essential to an efficient development of *Tilia cordata* somatic embryos. Somatic embryos of several species of Norway, white, and interior spruce that matured on ABA showed levels and accumulation of storage proteins similar to that of zygotic embryos, as compared to somatic embryos that had not matured on ABA (Dunstan *et al.*, 1998; Misra *et al.*, 1993). ABA typically acts in response to a stress, functioning as a messenger (Chandler and Robertson, 1994; Marcotte *et al.*, 1992; Rudus *et al.*, 2009; Weatherwax *et al.*, 1996) and possibly substitutes some light-dependant endogenous hormones (Zelena, 2000). Other authors, (Ammirato, 1977) reported that ABA and dark conditions promoted the formation of cotyledonary-staged embryos with fewer anomalies. This effect was also observed at the histological level, with ABA-treated embryos showing high disorganization when exposed to light and more organized structures in dark. Also embryo cells had plastids with little reserve accumulation. The effect of ABA is normally associated as inhibitor of precocious germination, functioning as promoter of an efficient maturation prior germination. Nevertheless, ABA interaction with light, though not as clear as verified for PEG, had different responses for dark and light conditions. It is well-known that ABA is light sensitive. The bio-active (+) cis, trans-ABA could change into trans, trans-ABA under light. (Kong and von Aderkas, 2007) report that the trans, trans-ABA is not a favourite form in cultures of spruce somatic embryos. Bhatia *et al.* (2008) refer the differential regulation of light and ABA signalling pathways by a regulatory gene (SHW1) in Arabidopsis. Despite the interaction mechanism is not fully known, it is clear in our results that ABA has a light dependent response.

Sucrose effect

Reducing sucrose concentration increased twice the final number of somatic embryos. However, changing sucrose concentration had little stimulatory effect on improving embryo quality and maturation/conversion rates relatively to control. Similar results were verified by Troch *et al.* (2009), where different carbohydrate sources, including sucrose, had no direct stimulatory effect. Nevertheless, in our study, as in Robichaud's *et al.* (2004), higher levels of sucrose increased embryo starch reserves. Sucrose plays a dual nutritional and osmoticum role, being the most common sugar in the phloem of Angiosperms (Zimmermann and Ziegler, 1975). Supporting the higher number of somatic embryos under lower sucrose

concentrations, some authors report starvation as an essential shock to the somatic embryogenesis process (Choi *et al.*, 1999; Lee *et al.*, 2001). Moreover, Charriere and Hahne (1998) showed that the concentration of sucrose is directly related with the absorption of 6-benzylaminopurine (BAP), auxins and cytokinins in sunflower explants, functioning as a switcher between organogenesis and embryogenesis, which can explain the low embryogenic development verified at high(er) concentration(s). The alteration of exterior osmotic potential due to alterations of sucrose concentration disclosed to promote some phenolic accumulation, although PEG addition did not have the same effect. However, as suggested by Reis *et al.* (2008b) for *Feijoa sellowiana* somatic embryos formed in the presence of phenolic compounds, these may not have the appropriate amounts of sucrose necessary for basic metabolic activities and therefore become arrested at the globular stage or even at earlier stages of embryo development, leading to no maturation. The increase of sucrose availability resulted in cells with a superior metabolic tax comparatively to the zygotic embryo, characterized by high number of mitochondria, plastids (in particular amyloplasts) and lipidic bodies. These facts might indicate a possible deficient maturation (where reserve accumulation plays a central role), probably due to an excess of osmotic potential in the culture medium.

Conversion

Keeping cork oak embryos in the same maturation conditions seems to be stressful and reduces conversion, in the case of PEG, or totally inhibits it, as observed for ABA. Contrarily, cold treatment reveals to be important to induce root/shoot formation, and seems essential to trigger germination/conversion. It is interesting to notice that cold is often reported as promoting germination in this and other species (e.g. Garcia-Martin *et al.*, 2001; Martinez *et al.*, 2008) even though always regarded as not good for maturation since it arrests metabolic processes necessary to embryo dedifferentiation. Several reports state the importance of chilling treatments in a mature stage of somatic embryos as essential to achieve conversion. Pond (2005) showed how conversion was improved by exposing plates of fully mature white spruce embryos to 5º C, or immature or mature embryos to 10º C, for a minimum of 4 weeks. Moreover, several studies in cork oak somatic embryogenesis report as essential a chilling treatment of 4º C to enhance conversion (Garcia-Martin *et al.*, 2001; Hernandez *et al.*, 2003a; Pintos *et al.*, 2008).

Conclusions

We demonstrate here that controlling the external conditions (namely by adding PEG) of cork oak maturation improves somatic embryo quality and increases further germination and conversion rates. Therefore, the SE protocol published previously (Lopes *et al.*, 2006; Pinto *et al.*, 2002) is presently improved by maturating somatic embryos in a PEG-containing medium during two months with light at 24ºC, followed by transfer to basal medium where they were submitted to a two months cold treatment in the dark. Moreover, we demonstrate that these conditions promote reserve accumulation profiles more similar to those found in the zygotic embryos (e.g. starch accumulation). Successful manipulation of maturation/germination conditions also provides valuable tools for further studies on controlling synchronization at these stages. We believe that these data support that by manipulating external factors cork oak embling conversion still may be improved at synchronization of both apex and root formation level aiming for large scale production.

References

Ammirato, P.V., 1977. Hormonal-Control of Somatic Embryo Development from Cultured-Cells of Caraway - Interactions of Abscisic-Acid, Zeatin, and Gibberellic-Acid. Plant Physiology 59, 579-586.

Attree, S.M., Moore, D., Sawhney, V.K., Fowke, L.C., 1991. Enhanced Maturation and Desiccation Tolerance of White Spruce [*Picea-Glauca* (Moench) Voss] Somatic Embryos - Effects of a Non-Plasmolysing Water-Stress and Abscisic-Acid. Annals of Botany 68, 519-525.

Attree, S.M., Pomeroy, M.K., Fowke, L.C., 1995. Development of White Spruce (*Picea-Glauca* (Moench) Voss) Somatic Embryos during Culture with Abscisic-Acid and Osmoticum, and Their Tolerance to Drying and Frozen Storage. Journal of Experimental Botany 46, 433-439.

Bhatia, S., Gangappa, S.N., Chattopadhyay, S., 2008. SHW1, a common regulator of abscisic acid (ABA) and light signaling pathways. Plant Signaling & Behavior 3, 862-864.

Cailloux, F., JulienGuerrier, J., Linossier, L., Coudret, A., 1996. Long-term somatic embryogenesis and maturation of somatic embryos in *Hevea brasiliensis*. Plant Science 120, 185-196.

Cangahuala-Inocente, G.C., Steiner, N., Maldonado, S.B., Guerra, M.P., 2009. Patterns of protein and carbohydrate accumulation during somatic embryogenesis of *Acca sellowiana*. Pesquisa Agropecuaria Brasileira 44, 217-224.

Capuana, M., Petrini, G., Di Marco, A., Giannini, R., 2007. Plant regeneration of common ash (*Fraxinus excelsior* L.) by somatic embryogenesis. In Vitro Cellular & Developmental Biology-Plant 43, 101-110.

Chalupa, V., 2005. Protocol of Somatic Embryogenesis: Pedunculate Oak (*Quercus robur* L.) and Sessile Oak (*Quercus petraea* /Matt./ Liebl.), Protocol for Somatic Embryogenesis in Woody Plants, pp. 369-378.

Chandler, P.M., Robertson, M., 1994. Gene-Expression Regulated by Abscisic-Acid and Its Relation to Stress Tolerance. Annu Rev Plant Phys 45, 113-141.

Charriere, F., Hahne, G., 1998. Induction of embryogenesis versus caulogenesis on in vitro cultured sunflower (*Helianthus annuus* L.) immature zygotic embryos: role of plant growth regulators. Plant Science 137, 63-71.

Choi, Y.E., Kim, J.W., Yoon, E.S., 1999. High frequency of plant production via somatic embryogenesis from callus or cell suspension cultures in *Eleutherococcus senticosus*. Annals of Botany 83, 309-314.

Dunstan, D., Dong, J.-Z., Carrier, D., Abrams, S., 1998. Events following ABA treatment of spruce somatic embryos. In Vitro Cellular & Developmental Biology - Plant 34, 159-168.

Elmaataoui, M., Espagnac, H., Michauxferriere, N., 1990. Histology of Callogenesis and Somatic Embryogenesis Induced Instem Fragments of Cork Oak (*Quercus Suber* L.) Cultured Invitro. Annals of Botany 66, 183-190.

Etienne, H., Sotta, B., Montoro, P., Miginiac, E., Carron, M.P., 1993. Relations between Exogenous Growth-Regulators and Endogenous Indole-3-Acetic-Acid and Abscisic-Acid in the Expression of Somatic Embryogenesis in *Hevea brasiliensis* (Mull Arg). Plant Science 88, 91-96.

FAO, 1996. Food and Agriculture Organisation. Country Report for Portugal. International Conference and Programme for Plant Genetic Resources, FAO, p. 15.

Fernandes, P., Rodriguez, E., Pinto, G., Roldan-Ruiz, I., De Loose, M., Santos, C., 2008. Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. Tree Physiology 28, 1841-1850.

Fernández-Guijarro, B., Celestino, C., Toribio, M., 1995. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell, Tissue and Organ Culture 41, 99-106.

Finchsavage, W.E., Clay, H.A., 1994. Evidence That Ethylene, Light-Acid and Abscisic-Acid Interact to Inhibit Germination in the Recalcitrant Seeds of *Quercus robur* L. Journal of Experimental Botany 45, 1295-1299.

Gaj, M.D., 2004. Factors Influencing Somatic Embryogenesis Induction and Plant Regeneration with Particular Reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulation 43, 27-47.

Garcia-Martin, G., Gonzalez-Benito, M.E., Manzanera, J.A., 2001. *Quercus suber* L. somatic embryo germination and plant conversion: Pretreatments and germination conditions. In Vitro Cellular & Developmental Biology-Plant 37, 190-198.

Hernandez, I., Celestino, C., Alegre, J., Toribio, M., 2003a. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis II. Plant regeneration from selected cork oak trees. Plant Cell Reports 21, 765-770.

Hernandez, I., Celestino, C., Toribio, M., 2003b. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Reports 21, 759-764.

Karkonen, A., 2000. Anatomical study of zygotic and somatic embryos of *Tilia cordata*. Plant Cell Tissue and Organ Culture 61, 205-214.

Kermode, A.R., 1995. Regulatory mechanisms in the transition from seed development to germination: interaction between the embryo and the seed environment, In: Kigel, J., Galili, G. (Eds.), Seed Development and Germination. Marcel Dekker, Inc, New York, pp. 273-332.

Kong, L., von Aderkas, P., 2007. Genotype effects on ABA consumption and somatic embryo maturation in interior spruce (*Picea glauca* x engelmanni). J. Exp. Bot. 58, 1525-1531.

Korbes, A.P., Droste, A., 2005. Carbon sources and polyethylene glycol on soybean somatic embryo conversion. Pesquisa Agropecuaria Brasileira 40, 211-216.

Krajnakova, J., Haggman, H., Gomory, D., 2009. Effect of sucrose concentration, polyethylene glycol and activated charcoal on maturation and regeneration of *Abies cephalonica* somatic embryos. Plant Cell Tissue and Organ Culture 96, 251-262.

Lee, E.K., Cho, D.Y., Soh, W.Y., 2001. Enhanced production and germination of somatic embryos by temporary starvation in tissue cultures of *Daucus carota*. Plant Cell Reports 20, 408-415.

Liao, Y.K., Amerson, H.V., 1995. Slash pine (*Pinus elliottii* Engelm.) somatic embryogenesis I. Initiation of embryogenic cultures from immature zygotic embryos. New Forests 10, 145-163.

Linossier, L., Veisseire, P., Cailloux, F., Coudret, A., 1997. Effects of abscisic acid and high concentrations of PEG on *Hevea brasiliensis* somatic embryos development. Plant Science 124, 183-191.

Lopes, T., Pinto, G., Loureiro, J., Costa, A., Santos, C., 2006. Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. Tree Physiology 26, 1145-1152.

Marcotte, W.R., Guiltinan, M.J., Quatrano, R.S., 1992. Aba-Regulated Gene-Expression - Cis-Acting Sequences and Trans-Acting Factors. Biochem Soc T 20, 93-97.

Martinez, T., Corredoira, E., Valladares, S., Jorquera, L., Vieitez, A.M., 2008. Germination and conversion of somatic embryos derived from mature *Quercus robur* trees: the effects of cold storage and thidiazuron. Plant Cell Tissue and Organ Culture 95, 341-351.

Mauri, P., Manzanera, J., 2004. Effect of abscisic acid and stratification on somatic embryo maturation and germination of holm oak (*Quercus ilex* L.). In Vitro Cellular & Developmental Biology - Plant 40, 495-498.

Misra, S., Attree, S.M., Leal, I., Fowke, L.C., 1993. Effect of Abscisic-Acid, Osmoticum, and Desiccation on Synthesis of Storage Proteins during the Development of White Spruce Somatic Embryos. Annals of Botany 71, 11-22.

Motoike, S.Y., Saraiva, E.S., Ventrella, M.C., Silva, C.V., Chamhum Salomao, L.C., 2007. Somatic embryogenesis of *Myrciaria aureana* (Brazilian grape tree). Plant Cell Tissue and Organ Culture 89, 75-81.

Murashige, T., Skoog, F., 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15, 473-497.

Nuutila, A.M., Kurten, U., Kauppinen, V., 1991. Optimization of Sucrose and Inorganic Nitrogen Concentrations for Somatic Embryogenesis of Birch (*Betula pendula* Roth) Callus-Cultures - a Statistical Approach. Plant Cell Tissue and Organ Culture 24, 73-77.

Ofei-Manu, P., Wagatsuma, T., Ishikawa, S., 2001. The significance of phenolic compounds in roots of different age on Al resistance of some common woody plants, pp. 512-513.

Ozyigit, I.I., Kahraman, M.V., Ercan, O., 2007. Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.). African Journal of Biotechnology 6, 3-8.

Park, Y.S., 2002. Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Annals of Forest Science 59, 651-656.

Pinto, G., Silva, S., Neves, L., Araujo, C., Santos, C., 2010. Histocytological changes and reserve accumulation during somatic embryogenesis in *Eucalyptus globulus*. Trees-Structure and Function 24, 763-769.

Pinto, G., Valentim, H., Costa, A., Castro, S., Santos, C., 2002. Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. tree. In Vitro Cellular & Developmental Biology-Plant 38, 569-572.

Pintos, B., Bueno, M.A., Cuenca, B., Manzanera, J.A., 2008. Synthetic seed production from encapsulated somatic embryos of cork oak (*Quercus suber* L.) and automated growth monitoring. Plant Cell Tissue and Organ Culture 95, 217-225.

Pond, S.E., 2005. The effect of temperature on conversion of white spruce somatic embryos. Propagation of Ornamental Plants 5, 35-44.

Puigderrajols, P., Mir, G., Molinas, M., 2001. Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in cork oak (*Quercus suber* L.). Annals of Botany 87, 179-189.

Pullman, G.S., Johnson, S., 2009. Osmotic measurements in whole megagametophytes and embryos of loblolly pine (*Pinus taeda*) during seed development. Tree Physiology 29, 819-827.

Rai, M.K., Jaiswal, V.S., Jaiswal, U., 2008. Effect of ABA and sucrose on germination of encapsulated somatic embryos of guava (*Psidium guajava* L.). Scientia Horticulturae 117, 302-305.

Rai, M.K., Jaiswal, V.S., Jaiswal, U., 2009. Effect of selected amino acids and polyethylene glycol on maturation and germination of somatic embryos of guava (*Psidium guajava* L.). Scientia Horticulturae 121, 233-236.

Reis, E., Batista, M.T., Canhoto, J.M., 2008a. Effect and analysis of phenolic compounds during somatic embryogenesis induction in *Feijoa sellowiana* Berg. Protoplasma 232, 193-202.

Reis, E., Batista, M.T., Canhoto, J.M., 2008b. Effect and analysis of phenolic compounds during somatic embryogenesis induction in *Feijoa sellowiana* Berg. Protoplasma 232, 193-202.

Robichaud, R.L., Lessard, V.C., Merkle, S.A., 2004. Treatments effecting maturation and germination of American chestnut somatic embryos. Journal of Plant Physiology 161, 957-969.

Rudus, I., Weiler, E.W., Kepczynska, E., 2009. Do stress-related phytohormones, abscisic acid and jasmonic acid play a role in the regulation of *Medicago sativa* L. somatic embryogenesis? Plant Growth Regulation 59, 159-169.

Sánchez-Romero, C., Márquez-Martín, B., Pliego-Alfaro, F., 2006. Somatic and Zygotic Embryogenesis in Avocado, In: Mujib, A., Šamaj, J. (Eds.), Somatic Embryogenesis. Springer Berlin / Heidelberg, pp. 271-284.

Shoemaker, R.C., Christofferson, S.E., Galbraith, D.W., 1987. Storage protein accumulation patterns in somatic embryos of cotton (*Gossypium hirsutum* L.). Plant Cell Reports 6, 12-15.

Shoji, M., Sato, H., Nakagawa, R., Funada, R., Kubo, T., Ogita, S., 2006. Influence of osmotic pressure on somatic embryo maturation in *Pinus densiflora*. Journal of Forest Research 11, 449-453.

Stasolla, C., van Zyl, L., Egertsdotter, U., Craig, D., Liu, W.B., Sederoff, R.R., 2003. The effects of polyethylene glycol on gene expression of developing white spruce somatic embryos. Plant Physiology 131, 49-60.

Tereso, S., Zoglauer, K., Milhinhos, A., Miguel, C., Oliveira, M.M., 2007. Zygotic and somatic embryo morphogenesis in *Pinus pinaster*: comparative histological and histochemical study. Tree Physiology 27, 661-669.

Toribio, M., Celestino, C., Gallego, F.J., Martinez, I., 1998. Induction of somatic embryogenesis in tissues from mature oak trees. COST 822, WG 1 'Physiology and control of plant propagation in vitro', meeting in Lisse, The Netherlands.

Troch, V., Werbrouck, S., Geelen, D., Van Labeke, M.C., 2009. Optimization of horse chestnut (*Aesculus hippocastanum* L.) somatic embryo conversion. Plant Cell Tissue and Organ Culture 98, 115-123.

Vila, S.K., Rey, H.Y., Mroginski, L.A., 2007. Factors affecting somatic embryogenesis induction and conversion in "Paradise tree" (*Melia azedarach* l.). Journal of Plant Growth Regulation 26, 268-277.

Weatherwax, S.C., Ong, M.S., Degenhardt, J., Bray, E.A., Tobin, E.M., 1996. The interaction of light and abscisic acid in the regulation of plant gene expression. Plant Physiology 111, 363-370.

Zelena, E., 2000. The effect of light on metabolism of IAA in maize seedlings. Plant Growth Regulation 30, 23-29.

Zimmermann, M.H., Ziegler, H., 1975. List of sugars and sugar alcohols in sieve-tube exudates, In: Zimmermann, M.H., Milburn, J.A. (Eds.), Transport in Plants. I. Phloem Transport. Berlin-Heidelberg, New York, pp. 480–503.

Chapter III

Genetic stability evaluation of *Quercus suber* L. somatic embryogenesis by RAPD analysis

Chapter section published as original article in SCI journal:

Fernandes P, Costa A, Rocha C, Santos C (2011) Genetic stability evaluation of *Quercus suber* L. somatic embryogenesis by RAPD analysis *Pakistan Journal of Botany*, 43(6): 2727-2731

Abstract

A reliable protocol for adult *Quercus suber* L. somatic embryogenesis (SE) was developed recently. To evaluate the potential use of this protocol in cork oak forest breeding programs, it is essential to guarantee somatic embryos/emblings genetic stability. Random Amplification of Polymorphic DNA (RAPD) is currently used to assess somaclonal variation providing information on genetic variability of the micropropagation process. In this work, SE was induced from adult trees by growing leaf explants on MS medium supplemented with 2,4-D and zeatin. Embling conversion took place on MS medium without growth regulators. DNA from donor tree, somatic embryos and emblings was used to assess genetic variability by RAPD fingerprinting. Fourteen primers produced 165 genetic loci with high quality and reproducibility. Despite somatic embryos originated some poor quality PCR-profiles, replicable and excellent fingerprints were obtained for both donor plant and embling. Results presented no differences among regenerated emblings and donor plant. Hence, the SE protocol used did not induce, up to moment, any genetic variability, confirming data previously obtained with other molecular/genetic techniques, supporting that this protocol may be used to provide true-to-type plants from important forestry species.

Keywords: cork oak, RAPD, somatic embryogenesis, stability.

Introduction

Quercus suber L. is a very important forest species, in terms of environment and economy, mostly in the Mediterranean. However, as a consequence of the oak population aging and susceptibility to environmental factors (e.g. fungus-host interactions, forest fires), a large percentage of cork oak populations are declining (Anon., 1996). Moreover, classical cork oak breeding programs are mostly conditioned by vegetative propagation limitations and by low rates of seed conservation. In vitro propagation is a valuable complement to classical breeding strategies (Santos *et al*., 2005) and provides an opportunity to develop clones with improved productivity or resistance (Brito *et al*., 2003; Santos *et al*., 2006).

It is well known that in vitro culture can induce somaclonal variation for example, mutation and/or epigenetic changes (Loureiro *et al*., 2007), which may hamper the implementation of clonal forestry programs or, on the counterpart, may provide interesting mutants. From all the in vitro techniques, somatic embryogenesis (SE) is the most promising method for clonal mass propagation, mostly because both root and shoot meristems are present.

In Q. suber, SE was achieved from several sources such as from leaves of seedlings (Fernández-Guijarro *et al*., 1995), zygotic embryos (Manzanera *et al*., 1993), and from leaf explants of juvenile or adult plants (Fernandes *et al*., 2008). However, for the inclusion of a SE-protocol in breeding programs, the quality (e.g. genetic variability) and performance of regenerated plants must be assessed (Conde *et al*., 2004).

Using Q. suber somatic embryos from several embryogenic lines obtained from young material (zygotic embryos), no somaclonal variation has been detected by Random Amplified Polymorphic DNA (RAPD) analyses (Gallego, 1997). This result was later confirmed for several embryogenic lines by Amplified Fragment Length Polymorphisms (AFLP) markers (Hornero *et al*., 2001). However, when using embryogenic lines from mature explants, AFLP analyses detected somaclonal variation in one genotype. Loureiro *et al*., (2005), using the SE protocol developed by Pinto *et al*., (2002) for leaves of mature cork oaks, found no ploidy or DNA content variations among somatic embryo lines. However, Lopes *et al*., (2006) using SSR markers found one mutation in one embryogenic line derived from an adult tree, while other adult or young-derived SE lines remained stable. These data suggest that the SE process, the age of explant and/or genotype influence the genetic stability. More, despite their reliability as markers, the individual information given by each marker is restricted.

Consequently, the information concerning genetic stability of this cork oak SE protocol must be confirmed by other markers together with continuous phenotypic evaluation. RAPD

analyses have been used as a reliable, quick and inexpensive method to identify clones and cultivars (Ali *et al*., 2009; Çelebi *et al*., 2009; Khatri *et al*., 2009) and to assess somaclonal variation (Valladares *et al*., 2006) . Therefore, the aim of this work was to evaluate the genetic stability of the Q. suber complete SE process, covering the mother plant, somatic embryos and resultant emblings, using RAPD fingerprinting.

Materials and methods

Somatic embryogenesis induction, maintenance and conversion

Somatic embryogenesis was induced using a modified (Pinto *et al*., 2002) protocol. Briefly, branches were collected during May and June from a 60-years-old Q. suber tree (QsG3) in the north of Portugal. Sprouted leaves were disinfected using ethanol (70%) and sodium hypochlorite (10%). Explants were placed on MS medium (Murashige & Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose, 2.5 $g.L^{-1}$ Gelrite® and 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9.0 µM zeatin. The pH was adjusted to 5.8. Explants were kept in the dark at 24±1ºC. Somatic embryos, when present, were isolated and transferred to MS medium without growth regulators (MSWH). Cultures were grown under a photoperiod of 16 hours and light intensity of 98±2 $\mu mol.m^{-2}.s^{-1}$. Every four weeks, somatic embryos were subcultured on fresh MSWH medium and maintained by repetitive somatic embryogenesis. Somatic white-opaque dicotyledonary embryos were isolated and transferred to MSWH for conversion and emblings then acclimatized to ex vitro conditions (Lopes *et al*., 2006). All chemicals used in these experiments were purchased from Duchefa (Haarlen, Netherlands).

DNA extraction and PCR amplification

DNA extraction and PCR amplification: For all experiments one genomic line was used (QsG3). For each sample genomic DNA was extracted from about 150 mg fresh material using DNeasy® Plant Mini Kit (QIAGEN™, Germany), according to the specifications of the supplier. DNA concentration and purity were estimated by 0.8% agarose gel electrophoresis stained with Ethidium bromide (EB) and compared with a standard molecular mass marker (lambda HindIII, NEB) by spectrophotometry at 260 and 280 nm (1 A_{260} Unit of dsDNA = 50µg mL^{-1} H_2O; Pure DNA: $A_{260}/A_{280} \geq 1.8$).

Amplifications were carried out in a Px2 Thermal Cycler. A total of forty decamer primers, primers 1 to 20 from kit C and 1-20 from kit S (Operon Technologies) were screened using 2 random samples each from regenerated plantlets, somatic embryos and donor tree for their

effective utilization in RAPD analysis of Quercus suber somatic embryogenesis (Rocha *et al*., 2006). The PCR volume was 25µL and contained: 25 to 50ng of template DNA, 100µM each dNTP, 200µM primer, 3mM $MgCl_2$ and 2 U of Stoffel fragment (Applied Biosystems, USA) in 1x reaction buffer (100mM Tris-HCl, 100mM KCl, pH 8.3). The thermocycler program consisted of a preliminary step of 2 min at 94ºC; 10 cycles of 30 sec at 94ºC, ramp of 1.5ºC.s^{-1} to reach annealing temperature, 1 min at 55ºC, a ramp of 1.5ºC.s^{-1} to reach 72ºC and 4.5 min at 72ºC; 25 cycles of 30 sec at 94ºC, a ramp of 1.5ºC.s^{-1} to reach annealing temperature, 1 min at 45ºC, a ramp of 1.5 min up to 72ºC and 4.5 min at 72ºC; a final step of 1 min at 72ºC. PCR products were stored at 4ºC until resolution by electrophoresis on 1.5% (w/v) agarose gels with EB staining, in 1xTBE (Tris-Borate-EDTA, pH 8.0), at 150V and room temperature.

PCR fragments were scored using the image analysis software GeneTools (Syngene, USA) on the basis of presence (1) or absence (0) of the amplified product and assembled in a data matrix. Profiles were compared using the Simple Matching coefficient. The software package SPSS for Windows (version 15.0, SPSS Inc.) was used for all calculations.

Results

RAPD analysis

Two sets of PCRs were carried out for RAPD fingerprinting of each sample. Only bands reproducible on all runs were considered for analysis. Each primer generated a unique set of amplification products ranging from 226 bp in OPC8 to 1910 bp in OPC9 in size. The number of bands for each primer varied from 2 in OPC18 to 10 for OPS12 (Table 5).

Fifteen primers used in this analysis yielded 165 scoreable bands, 92 for the OPC primers plus 73 fragments for the OPS primers (Table 5), with an average of 11 bands per primer. These 165 markers were monomorphic across all the samples. An example of this is shown in figure 4. The total number of reproducible scoreable bands was 990. No significant genetic variability among tested samples was detected with all 990 markers. Simple matching coefficient tests showed reproducible similarities above 95% (Table 6).

There are, in this species, some low intensity PCR products for somatic embryos. Despite these products seemed, at a first glance inexistent, the use of GeneTools for spectra analysis showed they were present (Figure 5). These faded bands are restricted to somatic embryos and are mainly due to poor quality of PCR-products for this type of material.

Table 5: Primers used in RAPD studies and number of reproducible scoreable bands for each primer.

Primer	Scoreable Bands
OPC1	7
OPC2	12
OPC3	9
OPC5	9
OPC8	15
OPC9	11
OPC14	10
OPC18	6
OPC19	13
OPS12	11
OPS14	12
OPS16	13
OPS17	11
OPS18	10
OPS19	16
Average	11

Discussion

Molecular markers (e.g. AFLP, microsatellites, RAPD, RFLP) had been used as reliable, powerful and quick tools in the analyses of somaclonal variation in somatic embryogenesis of both conifers and angiosperms (Leal *et al*., 2006; Burg *et al*., 2007; Lopes *et al*., 2009) and particularly in the Quercus genus (Hornero *et al*., 2001; Wilhelm *et al*., 2005; Valladares *et al*., 2006; Lopes *et al*., 2006; Fernandes *et al*., 2008). Sanchez *et al*., (2003), using 32 RAPD primers, found no intraclonal or interclonal polymorphism between embryogenic lines originated from the same seedling of Q. robur, concluding that these somatic embryos were genetically uniform. In the same study, no differences in DNA sequences were found between somatic embryos and the later converted emblings. Similarly, by RAPD analyses, Valladares *et al*., (2006) found no evidence of genetic variation within, or between, the embryogenic lines from three trees, or between these lines and the field tree.

Table 6: Similarity matrix for all generated fragments using 15 primers.

	Donor plant	**Somatic embryo**	**Converted plant**
Donor Plant	1,000	0,953	0,994
Somatic Embryo	-	1,000	0,959
Converted Plant	-	-	1,000

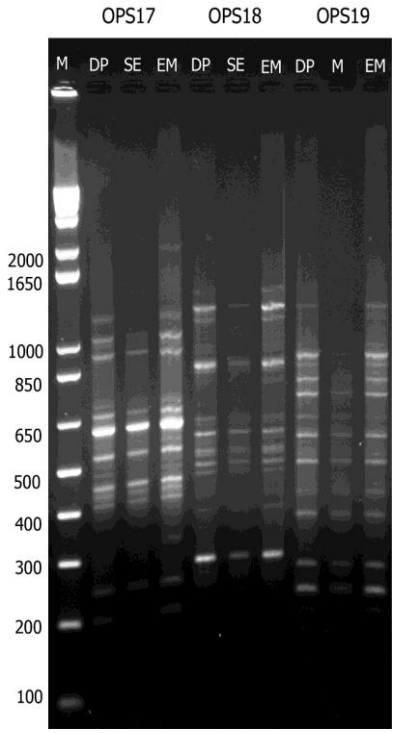

Figure 4: DNA profiles generated by the RAPD primers OPS 17, 18 and 19, in the three different stages of the somatic embryogenesis process: donor plant (DP), somatic embryo (SE) and embling (EM). M, size marker (1Kb Plus DNA Ladder).

In the specific case of somatic embryogenic lines of *Q. suber*, until the moment, either RAPD (Gallego, 1997), AFLP (Hornero *et al*., 2001; Fernandes *et al*., 2008) or microsatellites (Lopes *et al*., 2006) were used to evaluate somaclonal variation among somatic embryos within some embryogenic lines, and/or even among the explant leaves, embryos and emblings.

Studies on somatic embryogenesis genetic stability such as those of Loureiro *et al*., (2005) and of Lopes *et al*. (2006) are, in this paper, complemented with RAPD analyses to further validate this cork oak SE protocol. The choice of RAPD presents several advantages as it is an easy-of-use and development technique with a high quantity of information output. Also, the polymorphism level of RAPD analyses for different cork oak genotypes is referred by Gallego (1997) as high as 31.9%.

In our study, RAPD analyses do not show any somaclonal variation between the field tree, somatic embryos and the emblings obtained, using the modified somatic embryogenic protocol developed by Pinto *et al*. (2002). The total of 165 PCR products analyzed proved a perfect similarity between all the samples from all the three different somatic embryogenesis stages.

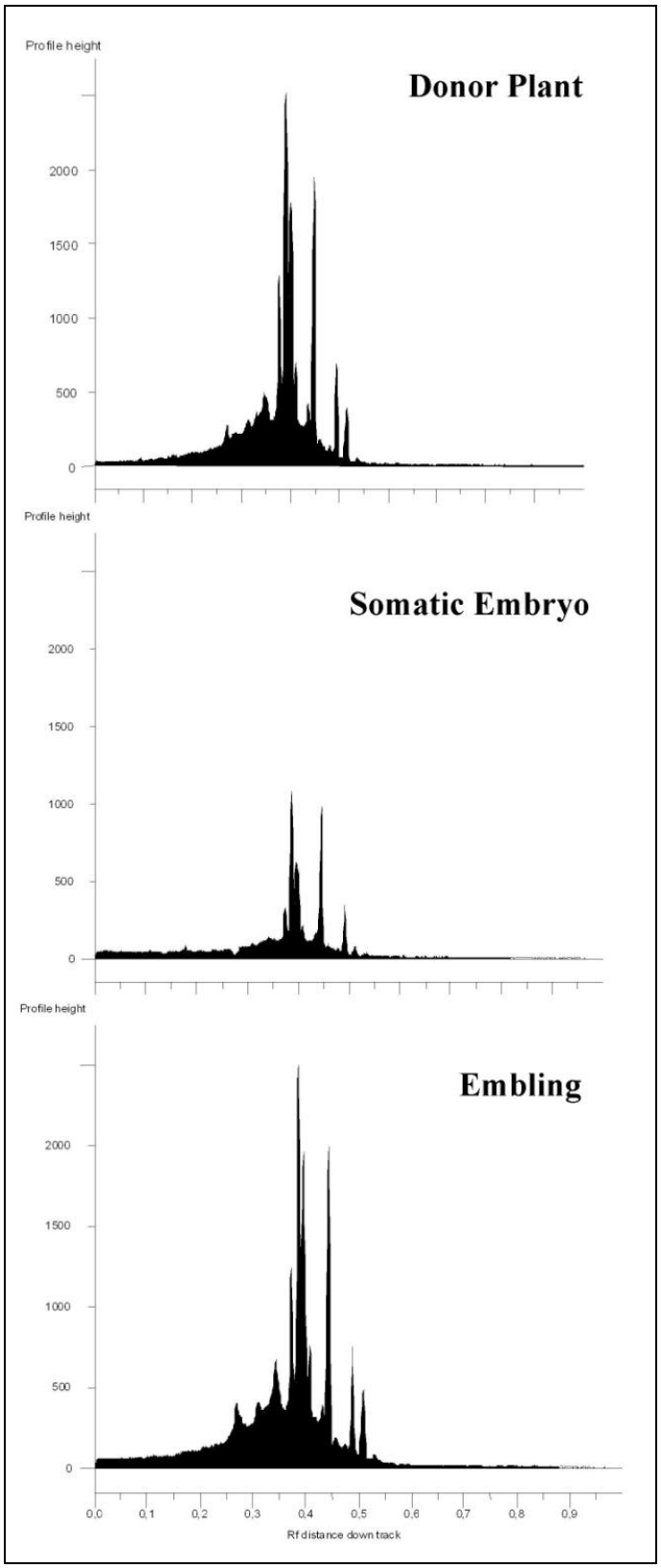

Figure 5: DNA profiles generated by the RAPD primer OPS 12, in the three different stages of the somatic embryogenesis process: donor plant, somatic embryo and embling.

Despite some reports of emblings regenerated from embryogenic callus cultures of e.g., date palm (Saker *et al*., 2000) have been found to exhibit somaclonal variation by RAPD analysis. Our study presents exactly the opposite. Emblings presented no differences compared to donor plant. However, somatic embryos showed lower band intensity (Figure 4). This fact could be due to the type of material and its high water content (>80%), which difficult amplification and further evaluation and scoring of PCR products. Nevertheless, despite the inherent importance of the process, since no variability was found at the converted plants, if there is any at the somatic embryo stage, it was, so far, not relevant. Also, Wilhelm *et al*. (2005), using microsatellites, found variation in Q. robur embryogenic lines but not in the regenerated plantlets. Since embryogenic cultures are exposed to plant growth regulators and suffer high cellular division rates, some somaclonal variation may occur. However, none of this putative variation was found at the emblings. This supports that the somatic embryogenic process did not induce changes in gene structure, which could significantly affect regenerated plantlets. Similar findings have been reported for *Q. serrata* (Thakur *et al*., 1999).

Our studies using SSR, FCM and now RAPD analysis show that our method is in general genetically stable and reliable. Yet, one must however not exclude the putative occurrence of other genetic changes (alteration in the DNA methylation, activation/inactivation of transposons and retrotransposons, activation/silencing genes, changing gene expression) (Gaj, 2004).

In conclusion, this work shows no genetic variability in plants obtained according to the modified available protocol, with respect to RAPD fingerprinting. Together with these results, those obtained by flow cytometry (Loureiro *et al*., 2005), microsatellites (Lopes *et al*., 2006) and morphological characterization of somatic embryos in this species (e.g. Pinto *et al*., 2002) support that our protocol used to somatic embryogenesis may be performed to provide true-to-type plants.

References

Ali, A., I. Khan, H. Ahmad and S. Ghafoor. 2009. Development of RAPD-based molecular markers for chromosome 5 of common wheat. Pak. J. Bot., 41(2): 691-694.

Anonymous. 1996. Food and Agriculture Organisation. Country Report for Portugal. In FAO International Conference and Programme for Plant Genetic Resources, p. 15.

Brito, G., A. Costa, H. Fonseca and C. Santo. 2003. Response of *Olea europaea* ssp. Maderensis In vitro shoots to osmotic stress. Scientia Horticulturae, 97: 411-417.

Burg, K., A. Helmersson, P. Bozhkov and S. von Arnold. 2007. Developmental and genetic variation in nuclear microsatellite stability during somatic embryogenesis in pine. J. Exp. Bot., 58: 687-698.

Çelebi, A., L. Açik and Z. Aytaç. 2009. Biosystematics studies among Ebenus species based on morphological, RAPD-PCR and seed protein analyses in turkey. Pak. J. Bot., 41(5): 2477-2486.

Conde, P., J. Loureiro and C. Santos 2004. Somatic embryogenesisand plant regeneration from leaves of *Ulmus minor* Mill. Plant Cell Reports, 22: 632-639.

Fernandes, P., E. Rodriguez, G. Pinto, I. Roldan-Ruiz, M. De Loose and C. Santos 2008. Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. Tree Physiology, 28: 1841-1850.

Fernández-Guijarro, B., C. Celestino and M. Toribio. 1995. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell, Tissue and Organ Culture, 41: 99-106.

Gaj, M. 2004.Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana*. Plant Growth Regulation, 43: 27-47.

Gallego, F., I. Martinez, C. Celestino and M. Toribio. 1997. Testing somaclonal variation using RAPD's in *Quercus suber* L. somatic embryos. Int. J. Pl. Sci., 158(5): 563-567.

Hornero, J., I. Martinez, C. Celestino, F.J. Gallego, V.Torres and M. Toribio. 2001. Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. Int. J. Pl. Sci., 162: 827-833.

Leal, F., J. Loureiro, E. Rodriguez, M.S. Pais, C. Santos and O. Pinto-Carnide. 2006. Nuclear DNA content of *Vitis vinifera* cultivars and ploidy level analyses of somatic embryo-derived plants obtained from anther culture. Plant Cell Reports 25: 978-985.

Lopes, T., A.Capelo, G. Brito, J. Loureiro and C. Santos. 2009. Genetic variability analyses of the somatic embryogenesis induction process in *Olea* spp., using nuclear microsatellites. Trees-Structure and Function, 23: 29-36.

Lopes, T., G. Pinto, J. Loureiro, A. Costa and C. Santos 2006. Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. Tree Physiology, 26: 1145-1152.

Loureiro, J., G.Pinto, T. Lopes, J. Dolezel and C. Santos 2005. Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L., using flow cytometry. Planta, 221: 815-822.

Loureiro, J., A. Capelo, G. Brito, E. Rodriguez, S. Silva, G. Brito and C. Santos. 2007. Micropropagation of *Juniperus phoenicea* L., adult plants and analyses of ploidy stability and DNA content in micropragation plants. Biologia Plantarum, 51: 7-14.

Manzanera, J.A., R. Astorga and M.A. Bueno. 1993. Somatic embryo induction and germination in *Quercus suber* L. Silvae Genetica, 42: 90-93.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Pinto, G., H. Valentim, A. Costa, S. Castro and C. Santos. 2002. Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. tree. In vitro Cellular & Developmental Biology-Plant, 38: 569-572.

Rocha, C., G. Pinto, F. Nóbrega, T. Lopes and C. Santos. 2006. Detection of histone H3 promoter genetic variability during *Quercus suber* L. embryogenesis. In: Book of Abstracts. XV National Congress of Biochemistry, 8-10 December, Aveiro, Portugal.

Saker, M.M., S.A. Bekheet, H.S. Taha, A.S. Fahmy and H.A. Moursy. 2000. Detection of somaclonal variations in tissue culture-derived date palm plants using isoenzyme analysis and RAPD fingerprints. Biologia Plantarum, 43: 347-351.

Sanchez, M., M. Martinez, S. Valladares and A. Vieitez. 2003. Maturation and germination of oak somatic embryos originated from leaf and stem explants: RAPD markers for genetic analysis of regenerants. J. Pl. Phys., 160: 699-707.

Santos, C., S. Fragoeiro and A. Phillips. 2005. Physiological response of grapevine cultivars and a rootstock to infection with *Phaeoacremonium* isolates: an In vitro approach using plants and calluses. Scientia Horticulturae, 103(2): 187-198.

Santos, C., S. Fragoeiro, H. Valentim and A. Phillips. 2006. Phenotypic characterization of *Phaeoacremonium* and *Phaeomoniella* strains isolated from grapevines. Scientia Horticulturae, 107(2): 123-130.

Thakur, R., S. Goto, K. Ishii and S. Mohan Jain.1999. Monitoring genetic stability in *Quercus serrata* Thunb. somatic embryogenesis using RAPD markers. Journal of Forest Research, 4: 157-160.

Valladares, S., C. Sanchez, M.T.Martinez, A. Ballester and A.M. Vieitez. 2006. Plant regeneration through somatic embryogenesis from tissues of mature oak trees: true-to-type conformity of plantlets by RAPD analysis. Plant Cell Reports, 25: 879-886.

Wilhelm, E., K. Hristoforoglu, S. Fluch and K. Burg. 2005. Detection of microsatellite instability during somatic embryogenesis of oak. Plant Cell Reports, 23: 790-795.

Chapter IV

Genotype is the most consistent factor affecting cell cycle dynamics during cork oak somatic embryogenesis

Chapter section submitted as original article in SCI journal:

Fernandes P, Rodriguez E, De Loose M, Santos C (2011) Genotype is the most consistent factor affecting cell cycle dynamics during cork oak somatic embryogenesis *Trees- Structure and Function* (submitted)

Abstract

Quercus suber L. is an economically and ecologically important species of the Mediterranean Basin. Its micropropagation is currently well established by somatic embryogenesis (SE), which results from an auxin/cytokinin ratio shock. Embryogenic callus may provide continuous source of somatic embryos by repetitive SE, however, during repetitive SE, these calli may dedifferentiate, losing their embryogenic ability. Embryogenic (EC) and non-embryogenic callus (NEC) lines from three elite cork oak diploid genotypes (QS0, QS3 and QS11) were analyzed by flow cytometry (FCM) to investigate putative differences in cell cycle dynamics. Both types of calli were grown on MS medium, containing different combinations of phytoregulators (PGRs): (i) 2,4-D (1 $mg.L^{-1}$); (ii) zeatin (2 $mg.L^{-1}$); and (iii) a combination of 2,4-D and zeatin (1 $mg.L^{-1}$ and 2 $mg.L^{-1}$, respectively). Cell cycle phase partitioning (G1:S:G2) were monitored for 4 weeks. Despite that PGRs and type of tissue had an influence in cell cycle distribution in punctual cases; the response induced was always genotype-dependent. We proved that different somatic embryogenesis responsive genotypes behave different through cell cycle progression during exposure to the same plant growth regulators used in SE induction.

Keywords: Cell cycle, flow cytometry, phytoregulators, somatic embryogenesis, repetitive embryogenesis

Introduction

Quercus suber L. is an economically and ecologically important species of the Mediterranean Basin, being the base for the cork transformation industry and it is best represented in Portugal (Costa *et al.*, 2008), making it the world largest cork producer. Due to the nature of its ecosystem, which have been subjected to alarming fires, namely since 2002 and especially in 2005, the area covered by this species has dramatically decrease. Consequently, it has become a necessity to outline and start programs that involve the regeneration of selected genotypes for both germplasm preservation and reforestation.

Somatic embryogenesis (SE) is frequently regarded as the best system for propagation of superior genotypes (Kim, 2000). Nonetheless, the loss of embryogenic and organogenic competence is one of the major problems when developing in vitro regeneration systems for propagation and manipulation of plants (Winklemann *et al.*, 1998). To be precise, during repetitive SE, calli may dedifferentiate and lose its embryogenic ability, leading to the production of two simultaneous types of calli under the same conditions: embryogenic (EC) and non-embryogenic (NEC) (Na *et al.*, 2007). These cellular masses (NEC) which rarely regain embryogenic ability are characterized by being mucilaginous, friable, and translucent or gray. NEC formation has been observed in SE of several species (Ikeda-Iwai *et al.*, 2002; Jimenez and Bangerth, 2001; Na *et al.*, 2007; Oinam and Kothari, 1995) and easily identifiable properties, such as: color, density and shape have been widely used as criteria to distinguish both types of calli (Shang *et al.*, 2009). This correlation between callus appearance and embryogenic competence has also been confirmed by histological studies (Quiroz-Figueroa *et al.*, 2002) and can be generalized for most embryogenic systems (Pasternak *et al.*, 2002).

For *Q. suber*, isolation of NEC from EC based on the above criteria proved NEC's incapability to produce any embryogenic structures (i.e. somatic embryos) in the same conditions than EC. Such abnormal tissue development is useless in micropropagation systems that employ embryogenesis (Na *et al.*, 2007). However it is interesting that two different types of calli occur under the same culture conditions (nutritional, hormonal and environmental), since cell competence largely relies on new signals (Dudits *et al.*, 1991). The developmental switch, leading a differentiated and resting cell state to a dedifferentiated, dividing, embryogenic state, likely involves the general reorganization of chromatin structure, overall reprogramming of gene expression, as well as cellular metabolism (Dudits *et al.*, 1991), causing a variation of cell cycle dynamics. Cytokinins and auxins, particularly, zeatin and 2,4-dichlorophenoxyacetic acid (2,4-D) are essential to induce somatic embryogenesis in *Q. suber*,

and are well known players of cell cycle regulation. Hormones involvement in regulating cell cycle could be through altering the kinetics of cell division (Francis and Sorrell, 2001), stimulating cell division and shortening cell cycles (Francis and Sorrell, 2001). Nonetheless, somatic tissues formation had also been reported in the absence of growth regulators (*e.g.* Choi *et al.*, 1998). Being so, the formation of two different types forms of calli from tissue placed in the same conditions, seems to point to different interpretation of stimuli and thus, different responses.

Despite the presently available protocol for SE induction works with most *Q. suber* genotypes, the reversion of EC into NEC and vice-versa is still poorly understood and often an uncontrolled process. In order to better understand the evolution of these two types of calli, the cell cycle partitioning for G0/G1:S:G2/M was evaluated considering the parameters: genotype and plant growth regulator (PGR).

Material and Methods

Initial plant material

All experiments used somatic embryogenic clusters (approximately 5 mm diameter in average) of *Quercus suber* at the globular stage induced as reported by Pinto *et al.* (2002). Three genotypes were used: QS0, QS3, and QS11. These genotypes were chosen based on their representative characteristics. QS0 is distinguished by high SE proliferation on MSWH (MS without growth regulators) medium, QS3 is characterized by high rates of plant conversion and QS11 is typified by elevated production of non embryogenic tissue (data not shown). All three genotypes are highly responsive to somatic embryogenesis induction according to Pinto *et al.* (2002) protocol.

For all three genotypes, two types of clusters were analyzed: embryogenic clusters (EC) and non-embryogenic clusters (NEC) based on their morphological (Figure 6) and responsiveness characteristics. EC was compact and brown, and embryos emerged from day 12 after induction, while NEC emerged both independently or from EC cultures around day 16 after induction; they were pale-yellow and did not give rise to somatic embryos.

All genotypes are currently maintained by repetitive embryogenesis at our lab for at least one year. These cultures were maintained in Petri dishes and stored in culture rooms with a constant temperature (23 ± 2 ºC) and controlled light conditions: 99 $\mu mol.m^{-2}.s^{-1}$ PAR (photosynthetic active radiation), photoperiod of 16 h, provided by Osram L: 36W/31830, cool white, fluorescent tubes. The plant material was subcultured every 4 weeks on standard solid MSWH (Murashige and Skoog, 1962) medium, supplemented with 30 $g.L^{-1}$ sucrose and

2.5 g.L^{-1} Gelrite® (control conditions). The pH of this standard medium was 5.8 prior to autoclaving (20 min at 121 ºC, 1bar). All plant culture products were acquired from Duchefa.

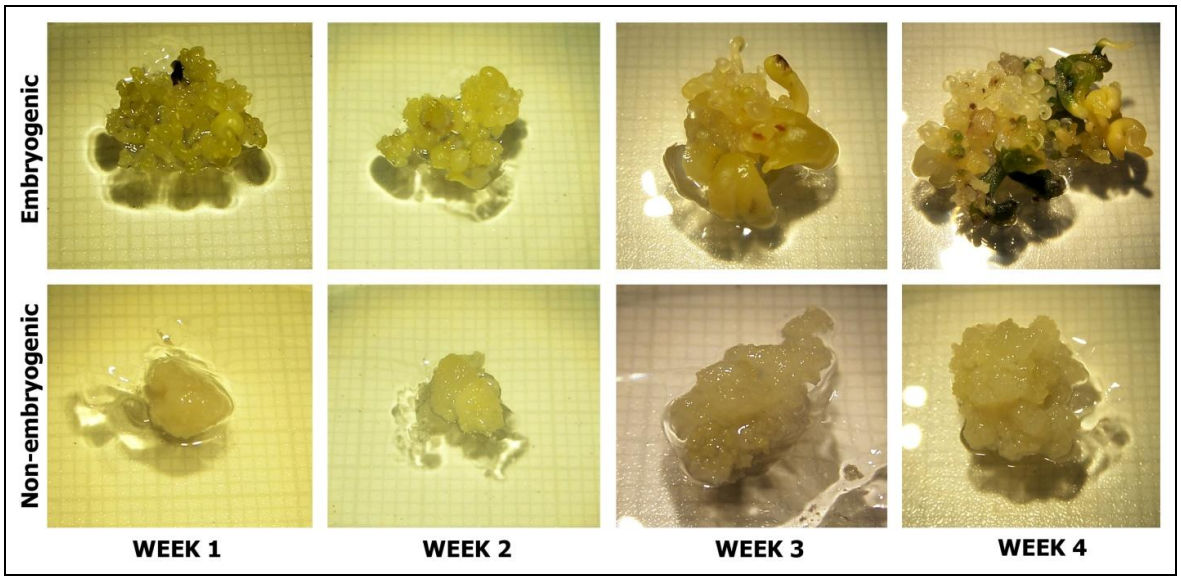

Figure 6: Embryogenic and non-embryogenic explants grown for four weeks in MSWH culture medium (control conditions).

Growth conditions

To assess growth regulators effect, globular-staged somatic embryos were isolated and placed to grow in standard MS supplemented with: i) 2 mg.L^{-1} zeatin, ii) 1 mg.L^{-1} 2,4-D and iii) 2 mg.L^{-1} zeatin plus 1 mg.L^{-1} 2,4-D (designated S6 medium). Control samples were grown in MSWH. All explants were kept in culture for four weeks in all conditions with at least three replicates.

Flow cytometric analyses

Somatic embryogenic clusters were analyzed by flow cytometry weekly, over 4 weeks, in order to assess putative changes in cell cycle dynamics. Samples were prepared as described by Loureiro *et al.* (2005) and Santos *et al.* (2007). In brief, the tissue was chopped with a razor blade in Woody Plant Buffer (Loureiro *et al.*, 2007) containing: 0.2 M Tris-HCl, 4mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabissulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5. The homogenate was then filtered through an 80-μm nylon mesh, to remove large debris and afterwards, 50 μg/ml of PI (Fluka) and 50 μg/ml of RNAse (Sigma) were added to each sample. After a 5 min incubation period at ice cold temperature, samples were analyzed in a Coulter EPICS-XL flow cytometer (Coulter Electronics, Hialeah,

Florida, USA) equipped with an air-cooled argon-ion laser (15 mW operating at 488 nm) and SYSTEM II software (v. 3.0, Beckman Coulter®). Before starting the analysis, the instrument was checked for linearity with fluorescent check beads (Coulter Electronics, Hialeah, FL) and the amplification was adjusted to position the sample nuclei peak at channel 200. This setting was kept constant throughout the analysis. The FL pulse integral versus FL pulse height and forward angle (FS) versus side angle (SS)-light scatter in logarithmic scale cytogram were used to define "interest zones" in order to separate intact nuclei from those that were broken or tagged with extraneous DNA or RNA, to eliminate doublets (events with a higher pulse area but the same pulse height as single nuclei) and to minimize the effects of cytosolic compounds (Brown *et al.*, 1991; Loureiro *et al.*, 2006a, b). For each type of condition (control, zeatin, 2,4-D and S6) 3 replicas were analyzed, being that at least 3000 nuclei were analyzed per sample.

Cell cycle evaluation and data analysis

For cell cycle analysis, flow cytometric files were transformed to single histograms files with WinMDI ver 2.8 software (Freeware by Joe Trotter of the Scripps Institute, La Jolla, CA.) and then analyzed using Cylchred software (Freeware developed by Terry Hoy of the University of Cardiff). Resultant data were treated using Microsoft Office Excel 2007. Statistical analyses were performed by running a One-Way ANOVA with a post-hoc Holm-Sidak test using SPSS (version 15.0 for Windows, SPSS Inc.)

Results

Cell cycle partitioning and dynamics of EC and NEC of three selected genotypes were followed and evaluated during four weeks. All calli were grown in four different conditions on MS medium, containing different combinations of PGRs in order to evaluate their importance in cork oak somatic embryogenesis, since selected PGRs are used for SE induction.

Results will be thoroughly dissected for each week, since significant variations occurred between and within all weeks. However, at week one no significant changes occurred at any level (data not shown) so results are presented from week two and forward. Three different parameters were evaluated as possible source of variation within all different cell cycle stages (PGRs, genotype and type of material) as well as their interaction affected G1:S:G2 partitioning.

Week 2

At week two, small differences were verified at both G1 and S levels. At these stages the only factor affecting the percentage of cells at those stages was genotype. For both G1 and S phases, genotype had a statistically significant (P<0.001) difference (Tables 6 and 7). A multiple comparison procedure indicated that, on one hand, QS11 had less cells arrested in G1 than QS0 and QS3 independently of PGR or type of material (EC or NEC), while on the other hand its S phase had more percentage of cells (Figures 7, 8 and 9). At the G2 level all factors had influence. Nevertheless, despite all factors influenced G1:S:G2 distribution, statistically significant interaction between PGRs, genotype and type of material (P=0.003) was clear (Table 8). Statistical analyses (detailed data not shown) revealed that the effect of the PGR/genotype interaction depends on which type of material is present (P<0.001). For both EC and NEC, PGRs leaded to different G2 percentages according to genotype: same PGRs have opposite effects for EC and NEC of the same genotype. While S6 incremented G2 cells in QS3 EC comparatively to QS0 and QS11, it had the opposite effect for NEC material. Same was verified for QS3 MSWH, comparatively to QS0 and QS11. In QS11, zeatin caused more cells to arrest at G2 after two weeks in NEC than in EC, regarding QS0 and QS3. Differences were also found for 2,4-D in both QS11 EC and NEC comparatively to QS0 and QS3 EC and NEC.

Table 7: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at G1-phase after 2 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	0.158	0.924
GENOTYPE	2	12.773	<0.001*
TYPE OF MATERIAL	1	2.895	0.093
PGR x GENOTYPE	6	1.712	0.130
PGR x TYPE OF MATERIAL	3	1.108	0.351
GENOTYPE x TYPE OF MATERIAL	2	1.220	0.301
PGR x GENOTYPE x TYPE OF MATERIAL	6	1.498	0.190

Week 3

At week three, some statistical relevant changes relatively to week two were found. At G1 stage, main differences were caused by PGRs and type of material. Contrarily to week 2, where only genotype had a clear influence, at week 3 all factors seemed to influence G1-staged percentage of cells. Regardless to other factors, type of material conditioned the distribution of cells arrested at G1 stage. At this checkpoint, in general, EC have more percentage of G1-staged cells than NEC. However, when we compare the influence type of

material within PGRs only zeatin had a significant difference (P=0.031). Nevertheless, the effect of different PGRs depended mostly on which genotype they were applied to.

Table 8: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at S-phase after 2 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	0.0440	0.988
GENOTYPE	2	12.916	<0.001*
TYPE OF MATERIAL	1	0.0437	0.835
PGR x GENOTYPE	6	1.440	0.210
PGR x TYPE OF MATERIAL	3	0.549	0.650
GENOTYPE x TYPE OF MATERIAL	2	2.420	0.096
PGR x GENOTYPE x TYPE OF MATERIAL	6	1.573	0.167

Table 9: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at G2-phase after 2 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	11.236	<0.001*
GENOTYPE	2	56.818	<0.001*
TYPE OF MATERIAL	1	122.988	<0.001*
PGR x GENOTYPE	6	1.204	0.314
PGR x TYPE OF MATERIAL	3	4.795	0.004*
GENOTYPE x TYPE OF MATERIAL	2	48.279	<0.001*
PGR x GENOTYPE x TYPE OF MATERIAL	6	3.720	0.003*

There is a statistically significant interaction between PGR and genotype (P=0.020) (Table 9). Within QS3, S6 culture media leaded to fewer cells in G1 than zeatin, 2.4-D and MSWH. Similar results were found for QS11. Zeatin and S6, which also contains zeatin, resulted as well in less G1 arrested cells. QS0 didn't present any susceptibility for PGRs at this point.

In what concerns S phase only one significant difference was found; only PGRs had a relevant effect, particularly, S6 lead to higher percentage of S-staged cells than control (MSWH) (Table 10). All other factors and conditions tested had no significant effect on S phase despite some trends might be visible (Figures 7, 8 and 9).

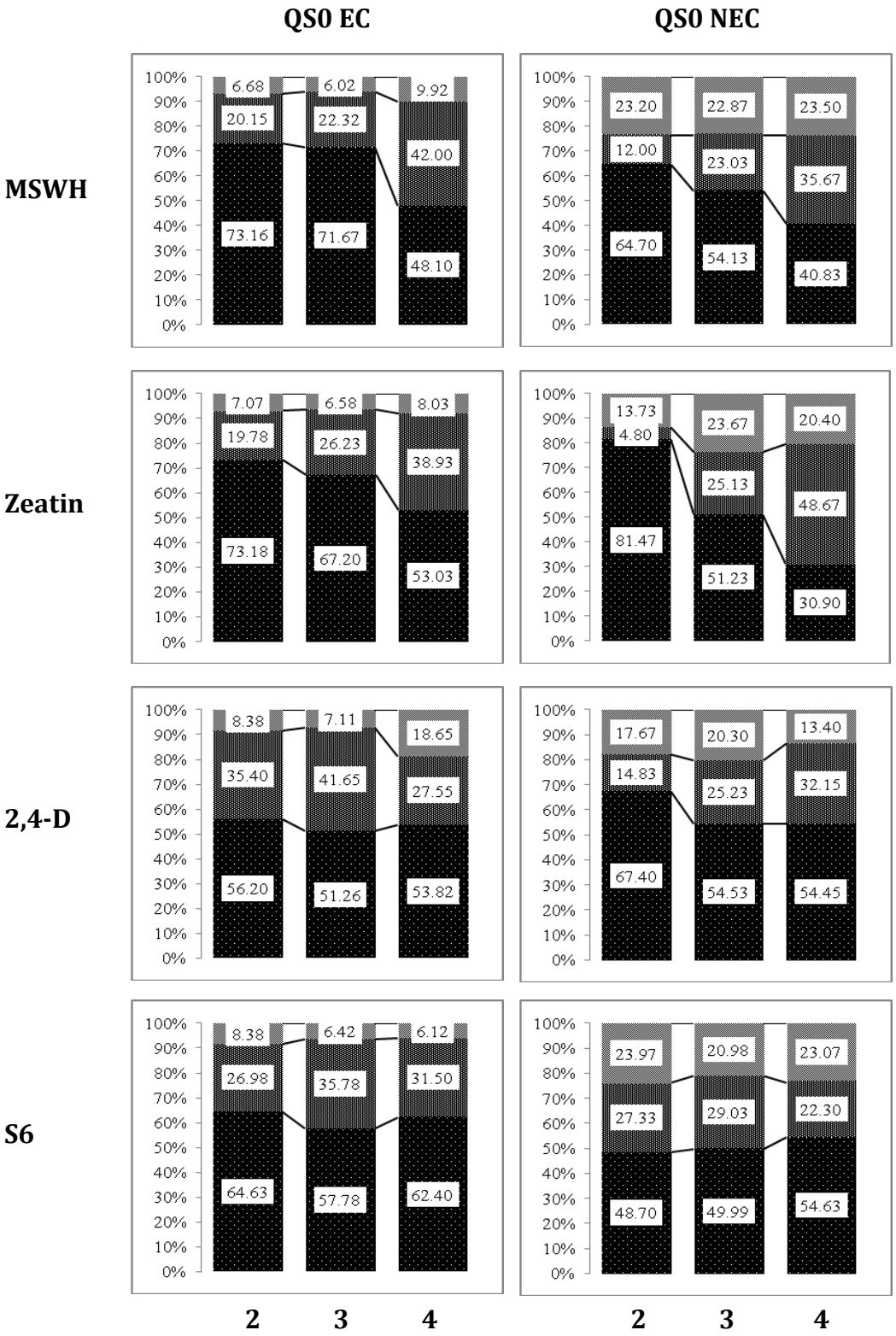

Figure 7: Embryogenic (EC) and non-embryogenic (NEC) clusters of QS0 genotype grown for four weeks (2, 3 and 4; week 1 not shown) in basal culture medium (MSWH), media supplemented with zeatin, 2,4-D and S6 culture medium. Images show distribution in percentage of nuclei among G0/G1 (black), S (dark grey) and G2 (light grey) mitotic stages.

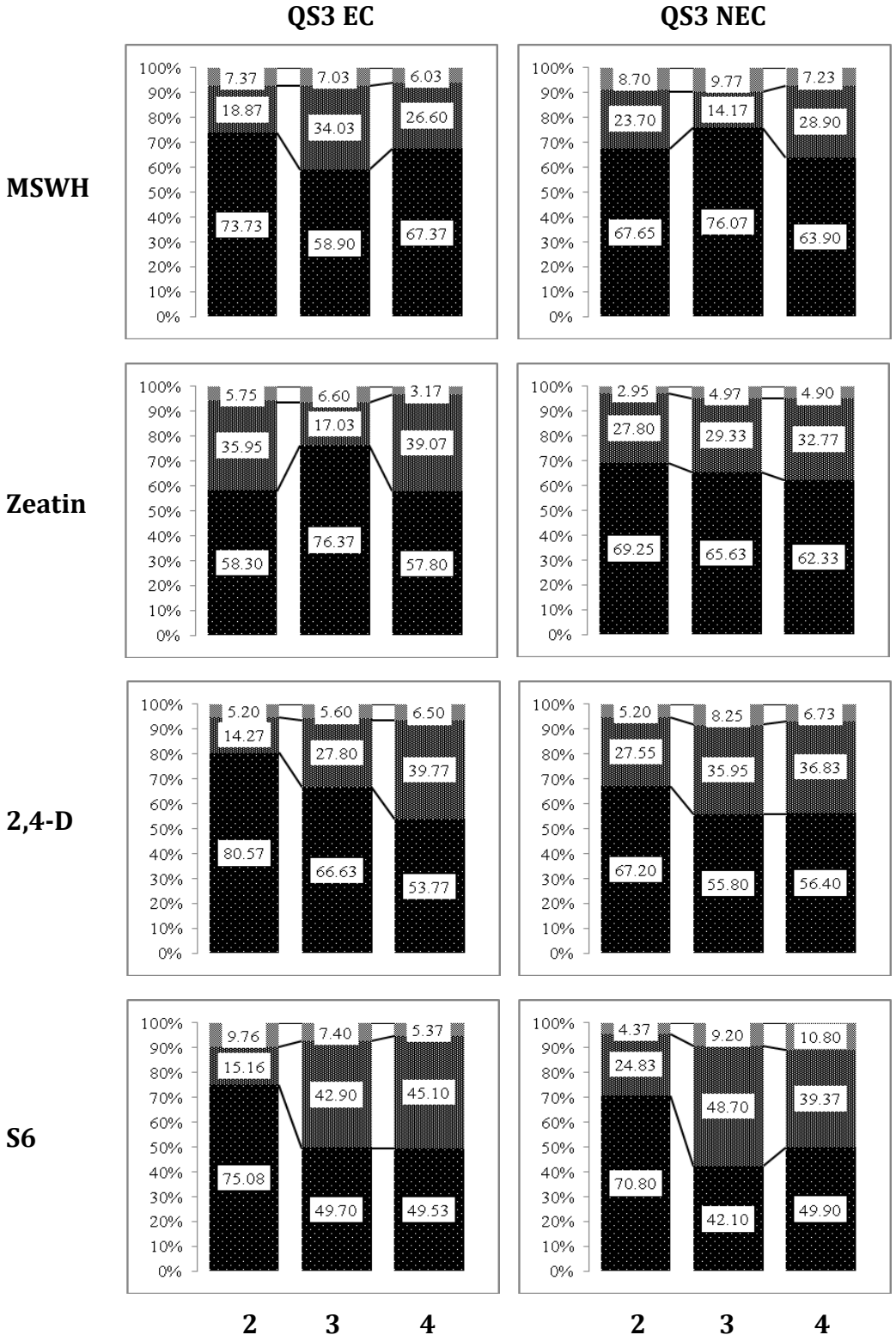

Figure 8: Embryogenic (EC) and non-embryogenic (NEC) clusters of QS3 genotype grown for four weeks (2, 3 and 4; week 1 not shown) in basal culture medium (MSWH), media supplemented with zeatin, 2,4-D and S6 culture medium. Images show distribution in percentage of nuclei among G0/G1 (black), S (dark grey) and G2 (light grey) mitotic stages.

Regarding G2 phase, very similar results to those obtained at week two were observed, despite some nuances. Once more the effect of the PGR/genotype interaction depended on what kind of material was present. There was a significant interaction for both EC and NEC ($P<0.001$) (Table 11). At this stage, PGRs had an influence within QS3 EC and QS11 EC only. In the former, control (MSWH) and S6 promoted a higher percentage of G2 cells, while in the later, zeatin had a stimulatory effect and 2,4-D had a inhibitory outcome. Same results were found for QS3 NEC and QS11 NEC. Once again QS0 suffered no significant alterations. Concerning genotype effect, results were different within same PGR and type of material. For zeatin EC, QS11 had more G2 cells, contrarily to week 2. Concerning 2,4-D EC, while no significant differences were found in week two, at week three QS11 has significantly fewer cells in G2 than G0 and QS3. This result is all the way around for 2,4-D NEC conditions. All other interactions had no significant results at this point (Table 11).

Table 10: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at G1-phase after 3 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	5.623	0.002*
GENOTYPE	2	0.779	0.462
TYPE OF MATERIAL	1	7.902	0.006*
PGR x GENOTYPE	6	2.698	0.020*
PGR x TYPE OF MATERIAL	3	0.623	0.602
GENOTYPE x TYPE OF MATERIAL	2	0.685	0.507
PGR x GENOTYPE x TYPE OF MATERIAL	6	1.420	0.218

Table 11: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at S-phase after 3 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	4.904	0.004*
GENOTYPE	2	0.330	0.720
TYPE OF MATERIAL	1	0.00101	0.975
PGR x GENOTYPE	6	2.160	0.056
PGR x TYPE OF MATERIAL	3	0.528	0.665
GENOTYPE x TYPE OF MATERIAL	2	1.463	0.238
PGR x GENOTYPE x TYPE OF MATERIAL	6	1.278	0.277

Table 12: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at G2-phase after 3 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	0.739	0.532
GENOTYPE	2	26.427	<0.001*
TYPE OF MATERIAL	1	108.953	<0.001*
PGR x GENOTYPE	6	2.166	0.056
PGR x TYPE OF MATERIAL	3	0.523	0.668
GENOTYPE x TYPE OF MATERIAL	2	29.650	<0.001*
PGR x GENOTYPE x TYPE OF MATERIAL	6	2.234	0.049*

Week 4

After four weeks, several changes occurred in cell cycle partitioning relatively to previous weeks. At this point, main significant differences are located at the G2 phase. Nevertheless, some relevant results were found at G1 and S stages. Despite that at G1 the effect of different levels of PGR depended on genotype (P=0.008) (Table 12) only QS0 was affected. Within MSWH, QS0 presented less percentage of cells at G1 than QS3 and QS11 for both EC and NEC. Moreover, those percentages were also lower within QS0 when comparing MSWH growing conditions with 2,4-D, zeatin and S6 ones (Figure 7).

Genotype QS0 had the exact contrary behavior at S stage. When cultured in MSWH, it had more cells held in S phase than QS3 and QS11. Also and only for QS0, MSWH resulted in higher percentage of cells at S phase than any PGRs (Figure 7). At this stage, the influence of type of material it is visible (Table 13), though this interdependence is significant only within S6, where EC has more S-staged cells than NEC (P=0.011) (Figures 7, 8 and 9). Type of material also behaved differently within genotypes. For QS11, regardless of the PGR used, EC always presented more cells at the S phase than NEC (Figure 9).

As for G2 stage, once again showed to be the most susceptible phase during somatic embryogenesis. After four weeks, a significant interaction between PGRs, genotype and type of material (P=0.017) was verified (Table 14). Comparisons for PGRs within QS3 EC and QS3 NEC showed that S6 culture conditions promoted more cells at G2 phase than other conditions (Figure 8) (Table 14). However, same analysis for QS11 EC revealed that 2-4-D had a significant inhibitory effect (P<0.001), while in NEC cultures 2,4-D and zeatin stimulated cells to G2 phase (Figure 9) (Table 14). Within PGRs and type of material, genotype had the more visible significant effect (P<0.001) (Table 14). For S6 EC and S6 NEC, QS3 presented fewer cells in G2 than QS0 and QS11.

Figure 9: Embryogenic (EC) and non-embryogenic (NEC) clusters of QS11 genotype grown for four weeks (2, 3 and 4; week 1 not shown) in basal culture medium (MSWH), media supplemented with zeatin, 2,4-D and S6 culture medium. Images show distribution in percentage of nuclei among G0/G1 (black), S (dark grey) and G2 (light grey) mitotic stages.

This result was also obtained for both MSWH EC and MSWH NEC. In this case, QS3 also presented fewer events in G2 than QS0 and QS11 (Figure 8). However, for 2,4-D, QS11 embryogenic cultures presented an inferior number of G2 nuclei than QS0 and QS3, while QS11 NEC had the opposite result (Figure 9).

Nevertheless, and despite that results were not always significant, there are some clear tendencies that might be relevant and will be further discussed. For QS0 there is a clear trend for S phase to increase along the time in detriment of G1 phase. Despite this tendency is visible for both QS0 EC and NEC, it is not verified for S6 culture conditions. Moreover, for QS11 this trend seems to happen all way around. For MSWH and zeatin, in both EC and NEC, S phase diminishes, while G1 and G2 increase. In QS3 cultures it is obvious a constant low G2 phase along the weeks, with only significant changes for S6, for which S phase increases enormously in detriment of G1, for both EC and NEC (Figures 7, 8 and 9).

Table 13: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at G1-phase after 4 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	0.136	0.938
GENOTYPE	2	2.291	0.108
TYPE OF MATERIAL	1	1.142	0.289
PGR x GENOTYPE	6	3.133	0.008*
PGR x TYPE OF MATERIAL	3	0.638	0.593
GENOTYPE x TYPE OF MATERIAL	2	1.120	0.331
PGR x GENOTYPE x TYPE OF MATERIAL	6	0.354	0.905

Table 14: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at S-phase after 4 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	0.157	0.925
GENOTYPE	2	1.211	0.304
TYPE OF MATERIAL	1	5.671	0.020*
PGR x GENOTYPE	6	4.346	<0.001*
PGR x TYPE OF MATERIAL	3	1.051	0.375
GENOTYPE x TYPE OF MATERIAL	2	0.275	0.761
PGR x GENOTYPE x TYPE OF MATERIAL	6	0.623	0.711

Table 15: Effect of plant growth regulators (zeatin, 2,4-D and S6), genotype and type of material (EC and NEC) in cork oak somatic embryos at G2-phase after 4 weeks. A three-way ANOVA followed by a Holm-Sidak test was used.

Source of Variation	DF	F	P
PGR	3	0.293	0.831
GENOTYPE	2	18.876	<0.001*
TYPE OF MATERIAL	1	56.887	<0.001*
PGR x GENOTYPE	6	1.637	0.149
PGR x TYPE OF MATERIAL	3	0.308	0.819
GENOTYPE x TYPE OF MATERIAL	2	6.816	0.002*
PGR x GENOTYPE x TYPE OF MATERIAL	6	2.777	0.017*

Discussion

The cell cycle partitioning stages of EC and NEC calli for three genotypes submitted to different plant growth regulators were characterized during four weeks. Cork oak calli were submitted to culture media supplemented with zeatin, 2,4-D and zeatin plus 2,4-D. The particular choice of these conditions was based on the fact that these are the PGRs used to induce somatic embryogenesis in adult cork oak leaves (Pinto *et al.*, 2002). As previously described by Pinto *et al.* (2002), growth regulator combinations in the induction medium were determinant for callus and somatic embryo induction. The addition of 9.0 μM zeatin to 4.5 μM 2,4-D was the only efficient treatment inducing embryogenic calluses subsequently producing large numbers of somatic embryos. Nevertheless, the role of both 2,4-D and zeatin in inducing somatic embryogenesis in *Quercus* species seems unclear. 2,4-D alone did not induce embryogenesis in *Q. suber* leaf explants according to Fernández-Guijarro *et al.* (1995) and Pinto *et al.* (2001; 2002). However, Bueno *et al.* (2000) described the contrary. Zeatin, despite that only Pinto *et al.* (2001; 2002) referred it as essential to induce cork oak somatic embryogenesis, it is widely described as fundamental in SE induction (Zhang *et al.*, 2009). Nevertheless the endogenous effects that both these auxin and cytokinin have in cork oak somatic embryogenesis cell cycle are not clear.

This study allowed us to obtain relevant information regarding cell cycle partitioning during somatic embryogenesis proliferation and infer about the effects of these PGRs on it.

Cell cycle dynamics suffered several changes during the four weeks. Most of the variations were resultant from interactions between the several PGRs, the type of material (EC or NEC) and genotype. Alterations were very punctual and though difficult to exploit, G1:S:G2 main differences were mainly due to genotype. Within each genotype clear trends were visible, which were not verified for the other genotypes. While for QS3, S6 seemed to increase S

phase in detriment of G1 phase along time, this was not visible for any of the other genotypes. For QS0 control, zeatin and 2,4-D alone have the same S-phase increment effect, in loss of G1 staged cells. However for QS11 the effect is clearly opposite. S phase diminishes and G2 increases significantly for MSWH, zeatin and 2,4-D. The stimulating effect of cytokinins/auxins on cell division has been observed in numerous cases ranging from the initiation of root primordia in planta to induction of cell division in protoplast suspension cultures (Houssa *et al.*, 1994; Raghavan, 2004). Françoise *et al.* (1998) points to zeatin as a highly specific triggering factor for G2-M transition in tobacco BY- 2 cells. Previously, Pascale *et al.* (1996) experiments showed an increase in zeatin levels during the G1 phase, following the decrease of the mitotic index. These results support an elevated number of cells arrested in G2, though type of plant material had an influence. It seems that the lack of embryogenic ability of NEC is due to deficiencies in cell cycle progression leading to no dedifferentiation. For instance, according to Label and Lelu (2000), embryogenic tissue in contrast to non-embryogenic callus, could not metabolize exogenous ABA in larch. This indicated that the ability of tissue to utilize exogenous ABA correlated with the capability of the tissue for somatic embryogenesis. This suggested lack of tissue ability could be inferred for our non-embryogenic cultures and so justify differences in tissue development. Continuous exposure to auxins/cytokinins is reported by some to arrest embryo development: Raghavan (2004) showed that reprogramming of cells of cultured zygotic embryos by 2,4-D is a critical first step in the induction of somatic embryos, although early-stage somatic embryos did not develop further during their continued growth in the auxin-containing medium. In fact, our control experiments did not present significant changes, probably due to the absence of auxins in culture media.

Nevertheless, despite all possible and described effects of auxins and cytokinins in somatic embryogenesis we prove here that genotype is the principal cause in responsiveness. Several reports refer to genotype as main variable in *Quercus* sp. somatic embryogenesis process. Hernandez *et al.* (2003) refer to genotype and time of harvesting, as well as their interaction, to significantly influence the frequency of induction of somatic embryogenesis. The influence of genotype on morphogenic processes is well documented for woody species, including the genetic control of somatic embryogenesis induced in zygotic embryos (Cheliak and Klimaszewska, 1991; Fernández-Guijarro *et al.*, 1995; Kim *et al.*, 1997; Park *et al.*, 1993) or in non-embryonic tissues (Barro *et al.*, 1999). A genotypic effect on the induction of embryogenesis in leaves from *Quercus robur* seedlings has also been suggested (Cuenca *et al.*, 1999). A strong genotypic effect on the ability to reprogram the cell fate of already differentiated cells into the embryogenic pathway has been observed (Wilhelm, 2000).

Significant variances in the embryogenic response due to families could be detected in *Q. acutissima* (Kim *et al.*, 1997) and *Q. suber* (Fernández-Guijarro *et al.*, 1995). (Toribio *et al.*, 1998) revealed the significant influence of individual adult cork oak trees on SE initiation frequency. This phenomenon is also reported by Cuenca *et al.* (1999), who were able to induce SE from stem and leaf explants of *Q. robur* from two different oak provenances out of four tested. Rancillac *et al.* (1996) explained the large variation in the induction ability (0±56%) of *Q. rubra* leaves from seedlings with the use of acorns of different geographic origin.

Androgenesis in *Q. petraea* (Jorgensen, 1988) was successful from only one genotype out of five different trees. In contrast, Elmaataoui and Espagnac (1987) used *Q. suber* leaves from two different seed sources for the induction of embryogenesis, but they did not observe any genotypic effect. The fact that different genotypes which respond to somatic embryogenesis induction PGRs, behave different through cell cycle progression during exposures to the exact same plant growth regulators might be an indicator of putative future mutations and/or somaclonal variations.

Our results are relevant for breeding and tissue culture purposes of cork oak because they will allow an early evaluation of the responsiveness trough a quick and efficient cell cycle analyses. This will speed up breeding programs and save effort by being able to predict embryogenic capacity of a line, based on the response of each genotype to an established induction protocol. In addition, high embryogenic capacity lines can be considered directly for genetic transformation or for massive multiplication for cork industries.

References

Barro, F., Canalejo, A., Martin, A., 1999. Genomic influence on somatic embryogenesis in the Triticeae. Plant Cell Reports 18, 769-772.

Brown, S.C., Devaux, P., Marie, D., Bergounioux, C., Petit, P.X., 1991. Flow-Cytometry - Application to Analysis of Plant Ploidy. Biofutur, 2-&.

Bueno, M.A., Gómez, A., Manzanera, J.A., 2000. Somatic and gametic embryogenesis in *Quercus suber* L., In: Gupta, P.K., Jain, S.M., Newton, R.J. (Eds.), Somatic Embryogenesis in Woody Plants. Kluwer Academic Publishers, Netherlands, pp. 479-507.

Cheliak, W.M., Klimaszewska, K., 1991. Genetic-Variation in Somatic Embryogenic Response in Open-Pollinated Families of Black Spruce. Theoretical and Applied Genetics 82, 185-190.

Choi, Y.E., Yang, D.C., Park, J.C., Soh, W.Y., Choi, K.T., 1998. Regenerative ability of somatic single and multiple embryos from cotyledons of Korean ginseng on hormone-free medium. Plant Cell Reports 17, 544-551.

Costa, A., Madeira, M., Oliveira, A.C., 2008. The relationship between cork oak growth patterns and soil, slope and drainage in a cork oak woodland in Southern Portugal. Forest Ecology and Management 255, 1525-1535.

Cuenca, B., San-Jose, M., Martinez, M.T., Ballester, A., Vieitez, A.M., 1999. Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Reports 18, 538-543.

Dudits, D., Bogre, L., Gyorgyey, J., 1991. Molecular and Cellular Approaches to the Analysis of Plant Embryo Development from Somatic-Cells Invitro. J Cell Sci 99, 473-&.

Elmaataoui, M., Espagnac, H., 1987. Neoformation of Somatic Embryos-Like Structures from Cork-Oak (*Quercus-Suber* L) Tissue-Cultures. Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences 304, 83-88.

Fernández-Guijarro, B., Celestino, C., Toribio, M., 1995. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell, Tissue and Organ Culture 41, 99-106.

Francis, D., Sorrell, D.A., 2001. The interface between the cell cycle and plant growth regulators: a mini review. Plant Growth Regulation 33, 1-12.

Françoise, L., Walter, D., Erwin, W., Marc Van, M., Dirk, I., Harry Van, O., 1998. Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. Febs Letters 426, 29-32.

Hernandez, I., Celestino, C., Toribio, M., 2003. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Reports 21, 759-764.

Houssa, C., Bernier, G., Pieltain, A., Kinet, J.M., Jacqmard, A., 1994. Activation of Latent DNA-Replication Origins - a Universal Effect of Cytokinins. Planta 193, 247-250.

Ikeda-Iwai, M., Satoh, S., Kamada, H., 2002. Establishment of a reproducible tissue culture system for the induction of *Arabidopsis* somatic embryos. Journal of Experimental Botany 53, 1575-1580.

Jimenez, V.M., Bangerth, F., 2001. Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis in vitro. Plant Science 160, 247-257.

Jorgensen, J., 1988. Embryogenesis in *Quercus petraea* and *Fagus sylvatica*. Journal of Plant Physiology 132, 638-640.

Kim, Y., 2000. Somatic embryogenesis in *Quercus acutissima*. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer, Dordrecht, 671-686.

Kim, Y.W., Youn, Y., Noh, E.R., Kim, J.C., 1997. Somatic embryogenesis and plant regeneration from immature embryos of five families of *Quercus acutissima*. Plant Cell Reports 16, 869-873.

Label, P., Lelu, M.A., 2000. Exogenous abscisic acid fate during maturation of hybrid larch (*Larix x leptoeuropaea*) somatic embryos. Physiologia Plantarum 109, 456-462.

Loureiro, J., Pinto, G., Lopes, T., Dolezel, J., Santos, C., 2005. Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry. Planta 221, 815-822.

Loureiro, J., Rodriguez, E., Dolezel, J., Santos, C., 2006a. Comparison of four nuclear isolation buffers for plant DNA flow cytometry. Annals of Botany 98, 679-689.

Loureiro, J., Rodriguez, E., Dolezel, J., Santos, C., 2006b. Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. Annals of Botany 98, 515-527.

Loureiro, J., Rodriguez, E., Dolezel, J., Santos, C., 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: A test with 37 species. Annals of Botany 100, 875-888.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

Na, H., Kim, K.W., Kwack, Y., Kim, S.K., Chun, C., 2007. Comparative anatomy of embryogenic and non-embryogenic calli from *Pimpinella brachycarpa*. Journal of Plant Biology 50, 344-350.

Oinam, G.S., Kothari, S.L., 1995. Totipotency of Coleoptile Tissue in Indica Rice (*Oryza sativa* L Cv Ch-1039). Plant Cell Reports 14, 245-248.

Park, Y.S., Pond, S.E., Bonga, J.M., 1993. Initiation of Somatic Embryogenesis in White Spruce (*Picea glauca*) - Genetic-Control, Culture Treatment Effects, and Implications for Tree Breeding. Theoretical and Applied Genetics 86, 427-436.

Pascale, R., Orit, S., Dirk, I., Marc Van, M., Harry Van, O., 1996. Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. Febs Letters 391, 175-180.

Pasternak, T.P., Prinsen, E., Ayaydin, F., Miskolczi, P., Potters, G., Asard, H., Van Onckelen, H.A., Dudits, D., Feher, A., 2002. The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiology 129, 1807-1819.

Pinto, G., Amaral, R., Santos, C., Carnide, O., 2001. Somatic embryogenesis in calluses of leaves from 3 years old *Quercus suber* L. plants. Quality enhancement of plant production through tissue culture. In: Book of Abstracts. Second Meeting of the Cost 843 WG3. Office for Official Publications of the European Communities, Carcavelos.

Pinto, G., Valentim, H., Costa, A., Castro, S., Santos, C., 2002. Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. tree. In Vitro Cellular & Developmental Biology-Plant 38, 569-572.

Quiroz-Figueroa, F., Méndez-Zeel, M., Sánchez-Teyer, F., Rojas-Herrera, R., Loyola-Vargas, V.M., 2002. Differential gene expression in embryogenic and non-embryogenic clusters from cell suspension cultures of *Coffea arabica*. Journal of Plant Physiology 159, 1267-1270.

Raghavan, V., 2004. Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. Am. J. Bot. 91, 1743-1756.

Rancillac, M., Klinguer, A., Klinguer, S., Millet, B., 1996. Preliminary investigations on somatic embryogenesis from leaf discs of red oak (*Quercus rubra* L). Plant Growth Regulation 20, 67-73.

Santos, C., Loureiro, J., Lopes, T., Pinto, G., 2007. Genetic fidelity analyses of in vitro propagated cork oak (*Quercus suber* L.), Protocols for Micropropagation of Woody Trees and Fruits, pp. 67-83.

Shang, H.H., Liu, C.L., Zhang, C.J., Li, F.L., Hong, W.D., Li, F.G., 2009. Histological and ultrastructural observation reveals significant cellular differences between agrobacterium transformed embryogenic and non-embryogenic calli of cotton. Journal of Integrative Plant Biology 51, 456-465.

Toribio, M., Celestino, C., Gallego, F.J., Martinez, I., 1998. Induction of somatic embryogenesis in tissues from mature oak trees. COST 822, WG 1 'Physiology and control of plant propagation in vitro', meeting in Lisse, The Netherlands.

Wilhelm, E., 2000. Somatic embryogenesis in oak (*Quercus* spp.). In Vitro Cellular & Developmental Biology-Plant 36, 349-357.

Winklemann, T., Sangwan, R.S., Schwenkel, H.G., 1998. Flow cytometric analyses in embryogenic and non-embryogenic callus lines of *Cyclamen persicum* Mill: relation between ploidy level and competence for somatic embryogenesis. Plant Cell Reports 17, 400-404.

Zhang, B.H., Wang, Q.L., Liu, F., Wang, K.B., Frazier, T.P., 2009. Highly efficient plant regeneration through somatic embryogenesis in 20 elite commercial cotton (Gossypium hirsutum L.) cultivars. Plant Omics 2, 259-268.

Chapter V

Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability

Chapter section published as original article in SCI journal:

Fernandes P, Rodriguez E, Pinto G, Roldan-Ruiz I, De Loose M, Santos C (2008) Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability *Tree Physiology* 28, 1841-1850.

Abstract

We describe a non-toxic encapsulation-dehydration procedure for the cryopreservation of cork oak (*Quercus suber* L.) somatic embryos that resulted in ≥ 90% survival. Genetic stability of the regenerated material was assessed by flow cytometry (FCM), amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR). Cryopreservation of embryogenic clusters involved encapsulation of each embryogenic cluster in an alginate bead, followed by a 3-day culture in 0.7 M sucrose and subsequent desiccation of the encapsulated embryogenic cluster to 25 or 35% water content (WC), followed by freezing in liquid nitrogen. Thawed cryopreserved somatic embryos had high recovery rates and exhibited long-term survival. No morphological differences were observed between somatic embryos desiccated to 25 and 35% WC. Analysis of DNA ploidy stability of control (*i.e.*, encapsulated-dehydrated but not frozen) and cryopreserved material by flow cytometry showed no significant differences. Similarly, DNA-marker analyses (AFLPs and SSR) revealed no significant differences between control and cryopreserved samples at the DNA-sequence level. Nonetheless, because polymorphisms were found between control material and samples cryopreserved and desiccated to 25% WC, the 35% WC method is recommended for cryopreservation of this tissue type. Cryopreservation of Q. suber somatic embryos by this non-toxic encapsulation-dehydration procedure is efficient and has potential for use in long-term conservation programs.

Keywords: AFLP, cork oak, FCM, somatic embryogenesis, SSR.

Introduction

Cryopreservation is currently applied to safeguard biodiversity and to store elite materials that have shown genetic superiority in field tests (Park, 2002), because it is highly efficient and the cryopreserved materials require minimal storage space (Bouman and De Klerk, 1990, Panis and Lambardi, 2005).

Cork oak (*Quercus suber* L.) is a species of major importance throughout the world, and particularly in southern Europe (Pinto *et al.,* 2002, Loureiro *et al.,* 2005). Cork oak conservation and breeding programs are constrained by limited natural regeneration, seed recalcitrance to conservation (Valladares *et al.,* 2004) and the fact that propagation by seed is not an option for true-to-type breeding of cork oak elite lines, because the seeds are not genetically identical to the seed-producing tree. Embryo cryopreservation provides an alternative method for storing species with recalcitrant seeds (Grout, 1986). González-Benito *et al.* (2002) demonstrated that the embryonic axes of cork oak are easily recoverable after cryopreservation. Furthermore, reliable protocols for the regeneration of *Q. suber* by somatic embryogenesis have been developed (e.g. Pinto *et al.,* 2002) that result in no or low rates of genetic variability (Loureiro *et al.,* 2005, Lopes *et al.,* 2006), thus providing a potential alternative to seed for cork oak breeding programs.

Many forest tree breeding programs have focused on the cryopreservation of somatic embryos of conifer species (e.g. Bomal and Tremblay, 2000); however, several techniques for the cryopreservation of somatic embryos of hardwood species have also been developed (see reviews by Engelmann, 2000, Takagi, 2000). For example, a protocol involving pre-treatment in sucrose-enriched medium followed by vitrification has been used successfully with embryogenic cultures of chestnut (Corredoira *et al.,* 2004) and *Q. robur* (Chmielarz *et al.,* 2005). Martinez *et al.* (2003) and Valladares *et al.* (2004) reported successful cryopreservation of embryogenic cultures of *Q. robur* and *Q. suber* by vitrification. Procedures based on the use of cryoprotectants (e.g., dimethyl sulfoxide), osmoticants (e.g., sorbitol) and controlled freezing rates have been used with hardwood embryogenic cultures (e.g. Vendrame *et al.,* 2001).

Vitrification and the use of cryoprotectants require complex and highly toxic solutions with high osmotic potential, e.g. PVS2 (Takagi, 2000), whereas the alternative encapsulation-dehydration method (Dereuddre *et al.,* 1991) makes use of non-toxic compounds although it requires expensive and complex apparatus if a slow-freezing technique is employed. The encapsulation-dehydration cryopreservation method has been used to preserve shoot tips

(e.g. *Robinia pseudoacacia*, Verleysen *et al.,* 2005a), in vitro suspension cells (e.g., *Medicago sativa*, Shibli *et al.,* 2001) and somatic embryos and embryogenic masses (e.g. *Coffea canephora*, Tessereau *et al.,* 1994; *Citrus* sp. Malik *et al.,* 2006; *Melea* sp. Scocchi *et al.* ,2007) but its use has never reported for the Fagaceae. Therefore, our first objective was to develop a non-toxic, inexpensive protocol for cryopreservation by encapsulation-dehydration for cork oak somatic embryos

Cryopreservation exposes the plant material to stressful conditions necessitating that the genetic fidelity of the thawed material be checked (see review by Panis and Lambardi, 2005). Therefore, our second objective was to evaluate the genetic fidelity of the cryopreserved somatic embryos after thawing based on three techniques—flow cytometry (FCM), amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR). Flow cytometry (FCM), which is frequently used to check the DNA ploidy stability of in vitro cultures of hardwood species (e.g., Conde *et al.,* 2004, Pinto *et al.,* 2004, Loureiro *et al.,* 2005, 2007, Leal *et al.,* 2006), was used to assess the genetic fidelity of the cryopreserved somatic embryos after thawing. In addition, we checked the occurrence of somaclonal variability based on amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR). The AFLP is sensitive multi-locus fingerprinting technique (Wilkinson *et al.,* 2003) that has been used to assess genetic diversity in the Quercus genus (Hornero *et al.,* 2001, Coart *et al.,* 2002, Ishida *et al.,* 2003) and to evaluate the genetic stability of cryopreserved material of hardwood species (Liu *et al.,* 2004). The microsatellite (SSR) marker technique has previously been used to evaluate genetic stability in in vitro cultures (Liu *et al.,* 2008) and in cork oak emblings (Lopes *et al.,* 2006) based on primers developed for other Quercus species (e.g., Isaghi and Suhandono 1997, Kampfer *et al.,* 1998, Steinkellner *et al.,* 1997).

Material and methods

Plant material and growth conditions

The study material was somatic embryogenic clusters (2.5 mm diameter on average) at the globular stage induced from a ~80-year-old *Quercus suber* (genotype G0) tree, as reported by Pinto *et al.* (2002). The embryogenic cultures had been maintained for 5 years in petri dishes in controlled-environment culture rooms with a constant temperature (23 ± 2 ºC) and a 16-h photoperiod at 99 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR), provided by Osram L: 36W/31830, cool white, fluorescent tubes. The plant material was sub-cultured every 4 weeks on standard solid MS medium (Murashige and Skoog, 1962), supplemented with 30 g l^{-}

^{1} sucrose and 2.5 g l^{-1} Gelrite®. The pH of the medium was 5.8 before autoclaving. All plant culture products were obtained from Duchefa.

Cryopreservation by encapsulation-dehydration

Somatic embryo clusters were isolated and subsequently encapsulated as described by Grout (1995). The encapsulation solutions, 0.1 M $CaCl_2$ solution and a 3% (w/v) alginate solution, were prepared in standard MS medium, to which 0.5 M sucrose was added before forming the beads. Embryo clusters were loaded in the 3% (w/v) alginate solution and then mixed with 0.1 M $CaCl_2$ solution to form beads with a diameter of 3–4 mm (each bead contained one embryogenic cluster). The encapsulated embryogenic clusters were incubated for 3 days in sucrose-enriched (0.7 M) MS liquid medium before being desiccated. For desiccation, beads were placed in open petri dishes and dried in the airflow of a laminar flow bench. Mass loss was monitored with an analytical balance and the water content calculated (Verleysen *et al.,* 2004). Two final water content (WC) values were chosen: 25% (CRY25) and 35% (CRY35), based on literature values (Niino and Sakai 1992, Hirata *et al.,* 1996, Gonzalez-Arnao *et al.,* 2000). For cryopreservation, beads were placed in cryotubes (10 per vial), frozen in liquid nitrogen (direct immersion) and stored for 24 h. Thawing was performed by immersing vials in a water bath (38 ºC for 2 min after which time no ice crystals were visible). Subsequently, the encapsulated explants were detoxified by incubation in 1.0 M sucrose in liquid MS medium for 1 h and the beads were then transferred to solid MS medium to support the growth of the somatic embryos. Controls (encapsulated and dehydrated to 25% and 35% WC) were processed as described above except that these somatic embryo clusters were not subjected to freezing and thawing.

Flow cytometry

Nuclear suspensions of somatic embryos were prepared according to Galbraith *et al.* (1983) as described by Loureiro *et al.* (2005). Briefly, the sample and the *Glycine max* cv. Polanka reference material (standard with 2C = 2.50 pg nuclear DNA, Doležel *et al.,* 1992; provided by Jaroslav Doležel, Institute of Experimental Botany, Olomouc, Czech Republic) were homogenized in Woody Plant Buffer (Loureiro *et al.,* 2007). The homogenate was filtered through 80-μm nylon mesh and then 50 μg ml^{-1} of propidium iodide (Fluka) and 50 μg ml^{-1} of RNAse (Sigma) were added. Samples were analyzed in a Coulter EPICS-XL flow cytometer (Coulter Electronics, Hialeah, FL). The 2C nuclear genome size (pg) of *Q. suber* was calculated as:

$$Q.\ suber 2\text{C nuclear DNA content (pg)} = \frac{Q.\ suber\ G_0/G_1\ \text{peak mean}}{G.\ max\ G_0/G_1\ \text{peak mean}} \times 2.50\ \text{pg}$$

Three replicates were analyzed per treatment (control, CRY25 and CRY35), and at least 5000 nuclei were analyzed per sample. To calculate total base pairs, we assumed that 1 pg of nuclear DNA contains 978 Mbp (Doležel *et al.,* 2003).

DNA extraction

Thawed somatic embryos that had been encapsulated-dehydrated and cryopreserved (CRY25 and CRY35) and non-frozen control somatic embryos were immersed in liquid nitrogen and lyophilized for 48 h. Dried material (20 mg) was ground and DNA was isolated according to the Qiagen extraction kit procedure. This method yielded up to 20 µg of genomic DNA per extraction. The DNA concentration was determined, relative to uncut lambda DNA, on 1.5% agarose gels.

AFLP assay

The AFLP assay was performed as described by Vos *et al.* (1995). Genomic DNA (288 ng) was digested for 2 h at 37 °C in a final volume of 25 µl containing 50 mM of MgAc, 250 mM of KAc, 50 mM Tris-HCl pH 7.5, 2.5 U of *Eco*RI (Invitrogen) and 2.5 U of *Mse*I (Invitrogen). Two adaptors, one for the *Eco*RI ends and one for the *Mse*I ends, designed to avoid the reconstruction of the restriction sites, were ligated to the restriction fragments by adding 24 µl of a mix containing 5 pmol of *Eco*RI adaptor, 50 pmol of *Mse*I adaptor, 10 mM of ATP, 1M of Tris-HCl, 1M of MgAc, 2M of KAc and 1 U of T4 DNA ligase (Invitrogen). The ligation mixture was incubated for 2 h at 37 °C. A pre-amplification step was performed with primers complementary to the *Eco*RI and *Mse*I adaptors with an additional selective 3′ nucleotide. The PCR analyses were performed in a 50 µl reaction volume containing 10× PCR buffer (Applied Biosystems), 5 mM of each dNTP, 25 ng of each primer (Invitrogen), 1.25 U of Taq DNA polymerase (Applied Biosystems) and 5 µl of the restriction products. The PCR amplifications (25 cycles) were carried out in a Perkin Elmer Geneamp PCR system 9600, each cycle consisting of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. For the selective amplification, 18 primer sets (*Eco*RI-XXX/*Mse*I-XXX) with three selective nucleotides were used, from which six were selected (Table 15) for further analysis, based on the level of polymorphism detected when tested with 10 genotypes (Table 16).

Table 16: Sequences of the primers used for selective AFLP amplification and the respective number of scored and polymorphic markers. Similarity between assays is also shown. Only polymorphisms marked with an asterisk (*) could be reproduced in a replicated experiment.

Name	Primer combination	Number of markers scored	Primer combinations + bp	Control	CRY25	CRY35
Q1	AAG/CAG	25	No polymorphisms were found			
Q2	ACA/CTG	7	No polymorphisms were found			
Q3	ACG/CAC	25	ACG/CAC 183*	0	1	0
			ACG/CAC 184	0	0	1
Q4	ACT/CAC	7	ACT/CAC 302	0	0	1
			ACT/CAC 326	0	0	1
Q5	AGC/CAA	32	AGC/CAA 98	1	0	0
			AGC/CAA 128*	0	1	0
			AGC/CAA 199*	0	1	0
Q6	AGC/CAG	22	AGC/CAG 63	0	1	1
			AGC/CAG 219	1	0	0
			AGC/CAG 323	1	0	0
			Similarity between pairs of samples			
			Control		0.941	0.941
			CRY25			0.949
			CRY35			

The *Eco*RI primer was labelled with one of three fluorescent dyes: HEX, FAM, NED. The PCR amplification mixture comprised 3 µl of diluted pre-amplification product (1/10 of the initial concentration), 1 µl of *Mse*I primer at 5 µM, 1 µl of *Eco*RI primer at 1 µM, 0.6 U of Taq DNA polymerase (Applied Biosystems), 2 µl of 10× PCR buffer (Applied Biosystems) and 0.2 µl of dNTPs (20 mM each; Invitrogen). The selective amplification was carried as follows: 1 cycle of 2 min at 94 °C, 30 s at 65 °C, 2 min at 72 °C, followed by eight cycles in which the annealing temperature decreased 1 °C per cycle, followed by 23 cycles of 1 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C. Samples were analyzed with an ABI Prism 3130 DNA sequencer (Applied Biosystems) using HEX, FAM, NED multiplexes. GeneScan Analysis Software 3.1.2 (Applied Biosystems) translated the information collected by the ABI3130 into fragment sizing information. In all cases, a peak amplitude threshold of 50 was set for the analysis. The GeneScan files were scored and recorded with GeneMapper 3.7 (Applied Biosystems), and a binary matrix was generated in Microsoft Excel. The AFLP bands were scored within the size range of 50–500 bp.

Table 17: Similarity matrix generated after AFLP fingerprinting for the six primer combinations listed in Table 1, with 10 cork oak genotypes.

Genotype	CL11	CL12	CL15	CL2	CL22	CL36	CL38	CL45	CL5	CL8
CL11	1.000	-	-	-	-	-	-	-	-	-
CL12	0.496	1.000	-	-	-	-	-	-	-	-
CL15	0.638	0.492	1.000	-	-	-	-	-	-	-
CL2	0.707	0.504	0.646	1.000	-	-	-	-	-	-
CL22	0.675	0.528	0.695	0.715	1.000	-	-	-	-	-
CL36	0.659	0.480	0.720	0.707	0.699	1.000	-	-	-	-
CL38	0.667	0.504	0.679	0.659	0.699	0.724	1.000	-	-	-
CL45	0.626	0.480	0.752	0.659	0.740	0.748	0.732	1.000	-	-
CL5	0.724	0.528	0.687	0.724	0.732	0.699	0.732	0.699	1.000	-
CL8	0.720	0.467	0.715	0.654	0.679	0.695	0.687	0.695	0.687	1.000

SSR assay

From the available nuclear microsatellites (nSSRs) developed for the *Quercus* genus, we chose four on the basis of their level of polymorphism (heterozygosity and number of alleles) and their PCR product quality. Briefly, of the four nSSRs assayed, two, AG46 and AG16, were first described by Steinkellner *et al.* (1997) for *Q. petraea* (Matts.) and the other two, MSQ4 and MSQ13 were developed for *Q. macrocarpa* (Dow *et al.,* 1995). For each DNA sample, PCR amplification of each of the four microsatellite loci was performed in a 20-µl reaction mix containing 5 ng of genomic DNA, 5 µl of 5X Colorless GoTaq® Flexi buffer (with 1.5 mM $MgCl_2$), 0.5 µl of 25 µM $MgCl_2$, 0.8 µl of 10 µM BSA, 0.5 µl of 5 µM dNTPs mix and 0.75 U of GoTaq DNA polymerase. Primer concentration was independently optimized for each primer pair. Details of the studied loci, respective primers, annealing temperature and sample concentrations are given in Table 17. Amplification of DNA was carried out in a Perkin Elmer Geneamp PCR system 9600 programmed as follows: 4 min at 94 ºC as the initial denaturing step, followed by 35 cycles of 94 ºC for 40 s, 40 s at each primer annealing temperature, and 72 ºC for 20 s. A final extension step was carried out at 72 ºC for 10 min. The forward primer was labelled with the Li-Cor infrared fluorescent dye IRD 700 or IRD 800. The PCR products were resolved on 6.5% denaturing polyacrylamide gels of 25 cm in length (Li-Cor KB-plus solution) along with Li-Cor labelled size standards (50–700 bp), and the band patterns were analyzed with an automated Li-Cor DNA Analyser Gene Readir 4200.

Table 18: Characteristics of the microsatellite loci used in this study. Repeat structure, primer sequences and variable PCR conditions are presented.

Locus	T_A (ºC)	Sample volume (µl)	Label	Range (bp)	Forward primer sequence	Reverse primer sequence
AG46[1]	50	1	IRD700	190-222	5′-CCCCTATTGAA GTCCTAGCCG-3′	5′-TCTCCCATGTA AGTAGCTCTG-3′
AG16[1]	55	1	IRD700	164-199	5′-CTTCACTGG CTTTTCCTCCT-3′	5′-TGAAGCCCTT GTCAACATGC-3′
MSQ4[2]	50	1	IRD800	203-227	5′-TCTCCTCTCCC CATAAACAGG-3′	5′-GTTCCTCTATCCA ATCAGTAGTGAG-3′
MSQ13[2]	50	1	IRD800	222-246	5′-TGGCTGCACCT ATGGCTCTTAG-3′	5′-ACACTCAGAC CCACCATTTTTCC-3′

[1] Steinkellner *et al.* (1997) for *Quercus petraea* (Matts.)
[2] Dow *et al.* (1995) for *Quercus macrocarpa.*

Data analysis

Each treatment was replicated three times and each replication consisted of one cryovial containing 10 beads. Appropriate controls (same protocol as used for cryopreservation but without freezing and thawing) were always included. Embryogenic clusters were regarded as surviving when new tissue was produced by Week 10 after thawing of the cryopreserved material. Statistical differences between controls and cryopreserved material, and between the CRY 25 and CRY35 cryopreservation protocols were evaluated by analysis of variance (ANOVA), in combination with the post-hoc Duncan or Tukey-Krammer multiple comparison test. The AFLP-marker profiles of controls and treatments were compared by the Simple Matching coefficient. The software SPSS for Windows v.13.0 (SPSS Chicago, IL) was used for all statistical calculations.

Results

Cryopreservation by encapsulation-dehydration

Comparison of recovery rates of thawed cryopreserved material with control material (encapsulated-dehydrated but not frozen) indicated that the cryopreservation protocol was highly efficient. Survival rates of the 25% and 35% WC controls (encapsulated-dehydrated but not frozen) were 100%. Ten weeks after recovery, both CRY25 and CRY35 had survival rates higher than 90% (Figure 10). There was no significant variation within and between groups ($P>0.05$). Recovered embryos presented similar white–opaque morphology as controls (Figure 11A and 11C). No delay or evident changes in proliferation rates were detected in recovered embryos compared with controls. Two weeks after transfer to proliferation medium, growth of somatic embryo clusters outside the beads was clearly

visible (Figure 11B and 11D). Conversion was followed by five months of culture on MS medium and no differences between cryopreserved and non-cryopreserved material were observed (Figure 12).

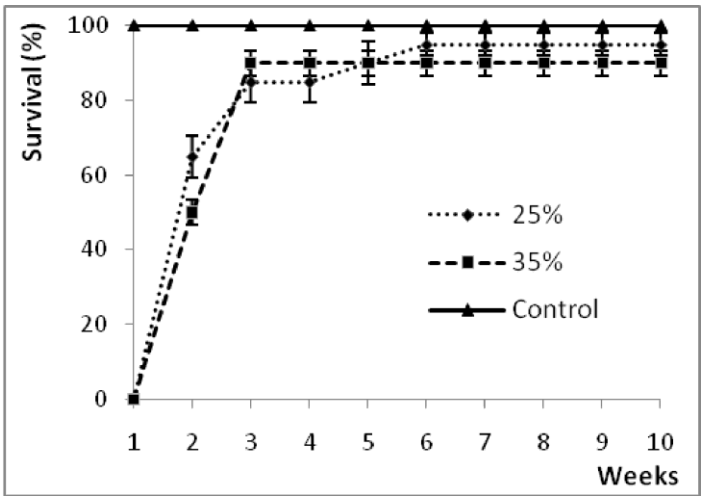

Figure 10: Survival percentages after 10 weeks of plant material subjected to the CRY25 and CRY35 protocols. Both controls (encapsulated-dehydrated but not frozen) had 100% survival, so only one line is represented. Vertical bars represent standard errors.

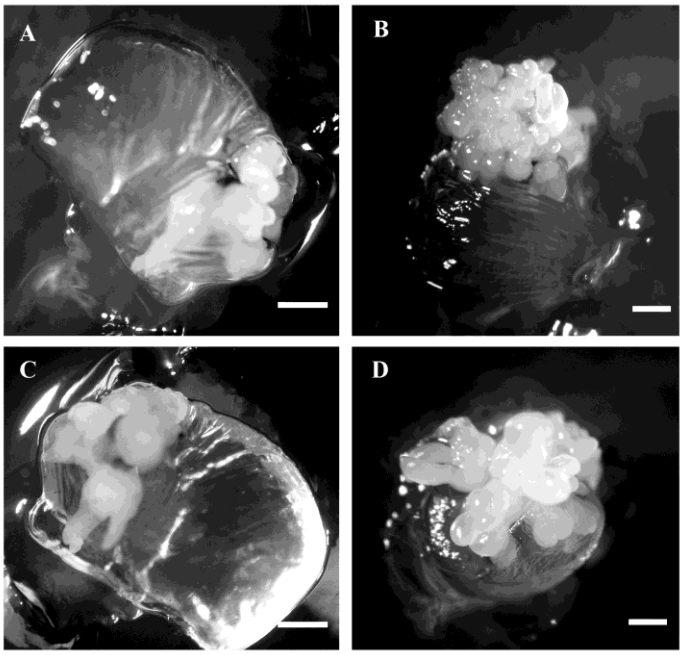

Figure 11: Cryopreserved and non-cryopreserved somatic embryo clusters. (A) Treated embryo cluster that was not cryostored; (B) treated embryo cluster that was not cryostored two weeks after recover; (C) treated embryo cluster that was cryostored after recovery treatment; and (D) treated embryo cluster that was cryostored two weeks after recovery. Bar = 1 mm

Figure 12: Plants derived from cryopreserved somatic embryos. The plants were obtained by spontaneous conversion of cotyledonary-stage somatic embryos. Bar = 1 cm.

Flow cytometry

All embryos analyzed by FCM provided reliable results, with low background and coefficients of variation (CV) below 5.0% (Figure 13). Control samples had the best performance, with CV=2.94%, whereas CRY25 had higher CV values of 3.70% (Table 18). The nDNA contents ranged from 2C = 1.93 pg DNA, for both controls and CRY35, to 2C= 1.94 pg DNA for CRY25. The minor differences in nDNA content between tissues were not statistically significant (P>0.05).

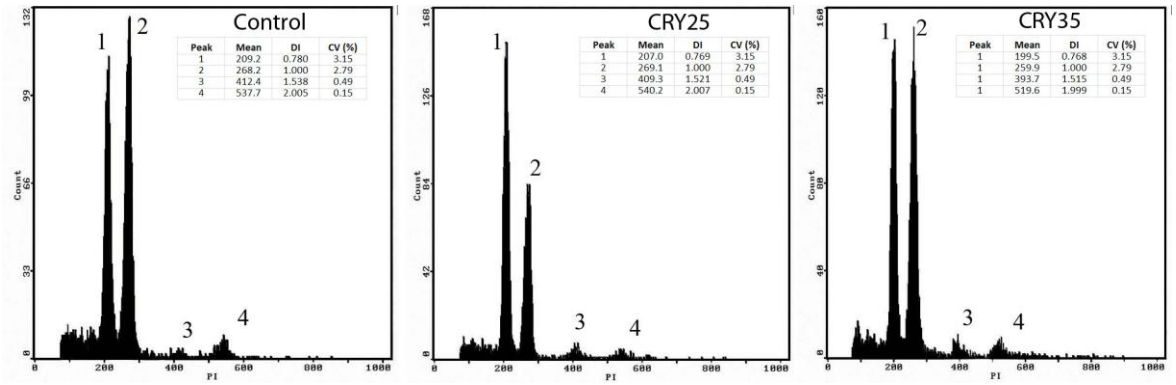

Figure 13: Plants derived from cryopreserved somatic embryos. The plants were obtained by spontaneous conversion of cotyledonary-stage somatic embryos. Bar = 1 cm.

Table 19: Nuclear DNA content of fresh and cryopreserved embryos of *Quercus suber*. Values are means and standard deviation (SD) of the 2C DNA content in mass values (pg). Nuclear DNA content in Mbp and mean coefficient of variation values obtained for the G_0/G_1 DNA peak (CV in %) are shown. Within the DI column, mean values followed by the same letter are not significantly different according to the Tukey-Kramer multiple comparison test at P≤0.01. Abbreviations and notes: 25% Cry and 35% Cry denote cryopreserved embryos desiccated to 25 and 35% water content, respectively. One pg DNA=978 Mbp (Dolezel *et al.*, 2003)

Tissue	DI	SD	2C DNA (pg)	SD	1c DNA (Mbp)	CV	n
Fresh	0.774[a]	0.0059	1.93	0.015	946	2.94	3
25% Cry	0.777[a]	0.0073	1.94	0.018	950	3.70	3
35% Cry	0.772[a]	0.0138	1.93	0.034	944	3.13	3

AFLP assay

The AFLP profiles were generated from frozen (CRY25 and CRY35) and non-unfrozen somatic embryo clusters, each constituting a single sample for the AFLP fingerprinting. Six AFLP primer combinations (Table 15) allowed us to screen 118 AFLP-markers (mean of 20 scorable bands per combination). The fingerprints of control, CRY25 and CRY35 were similar, with a mean similarity of 94%. The small discrepancy observed was caused by seven polymorphisms between control and CRY25 and between control and CRY35. Six AFLP-bands were polymorphic between CRY25 and CRY35 (Table 15). When the AFLP-profiles were generated again starting from the digestion/adaptor ligation step, higher similarities (96%, data not shown) were obtained. However, three DNA-banding polymorphisms, corresponding to 2.5%, were consistently found when new AFLP-fingerprints were generated. The primer combination E-AGC/M-CAA revealed two polymorphic fragments between CRY25 and control, and the primer combination E-ACG/M-CAC revealed one polymorphic band between CRY25 and control (Table 15 and Figure 14). The E-AGC/M-CAA polymorphic pattern was characterized by the presence of AFLP-bands at 128 and 199 bp in the CRY25 sample, which were absent in the control and in CRY35. For the primer combination E-ACG/M-CAC, an AFLP-band of 183 bp was found only in CRY25 but not in the control and CRY35 (Table 15 and Figure 14). However, the similarities detected between control, CRY25 and CRY35, about 0.94 (Table 15) were higher than those found between 10 cork oak genotypes, which had a similarity average of 0.66 (Table 16), based on the same set of AFLP-markers.

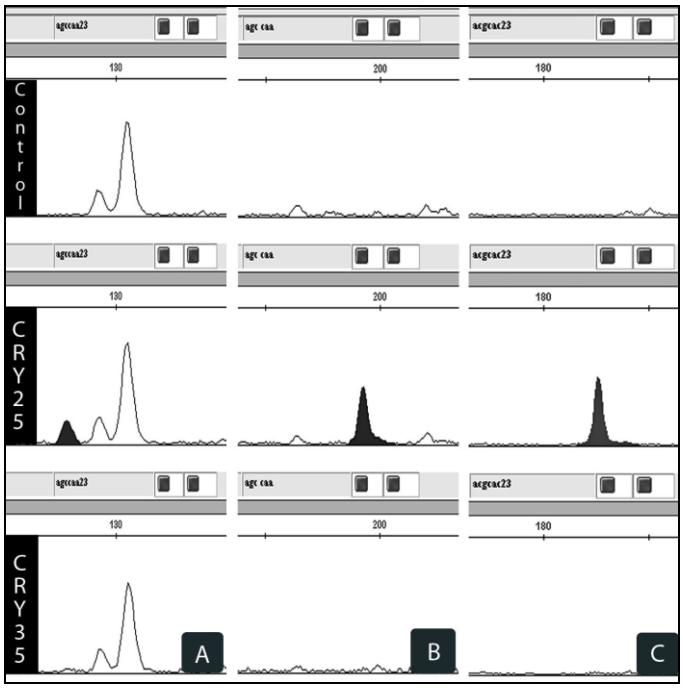

Figure 14: Three AFLP polymorphisms between CRY25 and control, confirmed in repeated reactions. Panels A and B show the two polymorphic bands generated with the primer combination E-AGC/M-CAA. Alleles were present only in the CRY25 sample, at 128 and 199 bp, respectively. Panel C illustrates one polymorphic fragment (182 bp) between CRY25 and the control, generated with the primer combination E-ACG/M-CAC.

SSR assay

The SSR analyses were performed in samples regenerated from frozen (CRY25 and CRY35) and control embryogenic clusters. Analysis of four SSR loci yielded a total of 18 reproducible alleles ranging in size from 175 to 228 bp. Allele sizes were well within the range of published values. All loci were heterozygous and no differences in banding pattern were observed between the treatments and control (Figure 15).

Figure 15: Amplification of MQS13 locus in control, CRY25 and CRY35 surviving explants. The pattern shows heterozygous individuals with two alleles of 228 and 217 bp (left to right) in lanes 1, 2 and 3.Standard fragment sizes in lane 4 are 255, 204 and 200 bp (left to right).

Discussion

We developed a simple and non-toxic encapsulation-dehydration technique to cryopreserve somatic embryos of cork oak that resulted in high recovery and genetic stability. Successful cryopreservation of somatic embryos from adult cork oak represents a major advance toward the use of somatic embryogenesis in future breeding programs of this species.

Encapsulation-dehydration was not toxic or stressful for the plant material, as indicated by the 100% survival rates of encapsulated-dehydrated but non-frozen samples. This method, unlike vitrification, does not require toxic cryoprotectants such as PVS2 (Volk *et al.*, 2006), thus minimizing stressful conditions. Similarly, for *Robinia pseudoacacia* cryopreservation, higher survival rates were obtained with encapsulation-dehydration (87%) compared with vitrification (78%) (Verleysen *et al.*, 2005a). Cryopreservation by encapsulation-dehydration has also been shown to be an efficient technique for somatic embryos of other woody species such as *Citrus* sp. (Gonzalez-Arnao *et al.*, 2003) and *Melia azedarach* (Scocchi *et al.*, 2007).

Both the 25% and 35% WC dehydration treatments resulted in high recovery of cork oak somatic embryos. Water content is a determinant parameter in cryopreservation experiments, because plant cells can be damaged by the formation of ice crystals during freezing or severe dehydration, or both (Engelmann 2000). The optimal WC is highly species dependent and can be defined as the value that allows freezing with minimal (or no) injuries to cellular components and guarantees the highest survival after cryopreservation: e.g., 33% for apple (Niino and Sakai, 1992), 38.6 % for azalea (Verleysen *et al.*, 2005b) and 19% for eucalypts (Pâques *et al.*, 1997). Clonal forestry strategies based on cryopreservation and in vitro culture (e.g., somatic embryos) may be advantageous. For example, Takagi *et al.* (1997) verified that cryopreserved taro shoot tips developed without intermediate callus formation, thus minimizing the risk of somaclonal variation usually associated with it. Cryopreservation may therefore reduce intermediate callus, an essential step in clonal conservation of germplasm (Takagi, 2000).

It is critical that embryogenic clones are efficiently maintained *sine die*, without change in genetic makeup or loss of viability during cryogenic storage (Park, 2002). Genetic instability may be produced during cryopreservation (Sakai, 2004); however, the number of studies on the occurrence of genetic variation in cryopreserved samples remains limited (*e.g.* Panis and Lombardi, 2005). For example, no genetic stability evaluations were performed in previously reported cryopreservation experiments using embryogenic cultures of *Q. suber* (Martinez *et al.*, 2003, Valladares *et al.*, 2004). However, Hao *et al.* (2002a) successfully cryopreserved cell suspensions of *Citrus* sp. without ploidy variation and randomly amplified polymorphic DNA

(RAPD) variations. In addition, surviving cells of some genotypes regenerated somatic embryos with better performance than the controls. Cryopreserved strawberry shoot tips also showed ploidy stability and AFLP analyses revealed only one band differing from non-cryopreserved samples (Hao *et al.*, 2002*b*). We assessed the genetic stability of recovered CRY25 and CRY35 material by a battery of techniques that give complementary information on ploidy levels, nDNA content, AFLP and SSR.

Flow cytometry confirmed the fidelity of the cryopreservation procedure. Both ploidy and DNA content were in concordance with published data: 2C = 1.90 pg DNA (Loureiro *et al.*, 2005, Santos *et al.*, 2007). Moreover, the variation in DNA content between control and cryopreserved samples was low (0.01 pg), as was the variation within a treatment. Although all CV values obtained were within the acceptance criteria for FCM samples (i.e. below 5%), as stated by Galbraith *et al.* (2002), small changes in DNA content may have occurred.

The typing of four SSR loci failed to detect any genetic variability, supporting our FCM results. The genotyping accuracy of the selected markers was previously tested in *Quercus* spp. (Dow *et al.*, 1995, Dow *et al.*, 1996, Steinkellner *et al.,* 1997). Samples were diploid and heterozygous as expected, and no polymorphisms were found between controls and treatments based on the resolution of this analysis with four microsatellite loci. Wilhelm *et al.* (2005) used two microsatellite loci, MSQ4, MSQ13, to assess genetic instability during somatic embryogenesis in *Q. robur* and detected variation among somatic embryos within all embryogenic lines, though no genetic instability was found among the regenerated plants. Lopes *et al.* (2006), who used other microsatellite loci, reported the occurrence of one mutation, representing a low mutation rate of 2.5%.

Genetic fingerprinting based on AFLP allowed direct analysis of variation at loci spread over the whole genome. No consistent polymorphisms were found between control and CRY35, indicating a perfect match between the control and CRY35 AFLP-fingerprints. The presence of three extra AFLP-bands in CRY25, which were not detected in the control sample or in CRY35, might be the result of putative small mutations, DNA methylation or cryo-selection of subpopulations (Müller *et al.*, 2007). Considering that the enzymes used for digestion are methylation-insensitive (Bonnema *et al.*, 2002), the possibility that DNA methylation could have caused these polymorphism is unlikely. Nevertheless, assuming that the three detected polymorphisms were the result of mutations caused by the cryopreservation procedure, the mutation rate was (2.5%). Hornero *et al.*, (2001) reported polymorphism values ranging from 5.6 to 7.3% between cork oak plants and embryogenic lines, which reinforces the suggestion that the consistent polymorphisms between control and CRY25 (2.5%) were the result of small differences at the DNA-sequence level. Furthermore, our data indicate that dehydration

to 25% WC was too stressful for the tissues, whereas dehydration to 35% WC induced no detectable genetic variation, supporting the use of 35% WC as the recommended dehydration value for *Q. suber* tissues. Analysis of a large number of regenerants, from the same or from other cork oak genotypes, will provide further insight into the frequency and relevance of these findings at the phenotypical level.

The regenerated plants appeared similar to plants obtained from acorns, presenting a dominant shoot apex, a well-developed stem and green leaves. Although we have reported previously genetic stability of regenerated plants from somatic embryogenesis (e.g. Loureiro *et al.*, 2005, Lopes *et al.*, 2006), DeVerno *et al.*, (1999) found variation in RAPDs among embryogenic *Picea glauca* (Moench) Voss cultures recovered from cryostorage, but not in the normal-appearing regenerated trees from those lines. Therefore, we are currently growing on our regenerated plan so that they can supply material for further genetic and phenotypic analyses.

In conclusion, despite the similar survival rates of the CRY25 and CRY35 cryopreservation protocols tested, the genetic stability data indicate that the CRY35 protocol is the most suitable for cryopreservation of cork oak somatic embryos. We demonstrated that cryopreservation of cork oak somatic embryos by encapsulation-dehydration is an efficient method of storage and the method is now being applied to create a germplasm bank of elite Portuguese cork oak trees to further evaluate its potential and to optimize plant regeneration. By combining the advantages somatic embryogenesis and cryopreservation, a considerable advance may be achieved in cork oak breeding programs.

References

Bomal, C. and F.M. Tremblay. 2000. Dried cryopreserved somatic embryos of two *Picea* species provide suitable material for direct plantlet regeneration and germplasm storage. Ann. Bot. 86:177–183.

Bonnema, G., P. Berg and P. Lindhout 2002. AFLPs mark different genomic regions compared with RFLPs: a case study in tomato. Genome 45:217–221.

Bouman, H.and G.J. De Klerk. 1990. Cryopreservation of lily meristems. Acta Hortic. 266:331–337.

Chmielarz, P., G.G.-d. March, M.-T. de Boucaud and se 2005. Cryopreservation of *Quercus robur* L. embryogenic calli. Cryoletters 26:349–355.

Coart, E., V. Lamote, M. De Loose, E. Van Bockstaele, P. Lootens and I. Roldan-Ruiz. 2002. AFLP markers demonstrate local genetic differentiation between two indigenous oak

species [*Quercus robur* L. and *Quercus petraea* (Matt.) Liebl] in Flemish populations. Theoret. Appl. Genet. 105:431–439.

Conde, P., J. Loureiro and C. Santos 2004. Somatic embryogenesis and plant regeneration from leaves of *Ulmus minor* Mill. Plant Cell Rep. 22:632–639.

Corredoira, E., M.C. San-Jose, A. Ballester and A.M. Vieitez 2004. Cryopreservation of zygotic embryo axes and somatic embryos of European chestnut. Cryoletters 25:33–42.

Dereuddre, J., S. Blandin and N. Hassen. 1991. Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen .1. Effects of preculture. Cryoletters 12:125–134.

DeVerno, L.L., Y.S. Park, J.M. Bonga, J.D. Barrett and C. Simpson 1999. Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss.]. Plant Cell Rep. 18:948–953.

Dolezel, J., J. Bartos, H. Voglmayr and J. Greilhuber. 2003. Nuclear DNA content and genome size of trout and human. Cytometry Part A. 51A:127–128.

Dolezel, J., S. Sgorbati and S. Lucretti. 1992. Comparison of 3 DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol. Plant. 85:625–631.

Dow, B.D. and M.V. Ashley. 1996. Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa* L. Mol. Ecol. 5:615–627.

Dow, B.D., M.V. Ashley and H.F. Howe. 1995. Characterization of highly variable (Ga/Ct)(N) microsatellites in the bur oak, *Quercus Macrocarpa* L. Theoret. Appl. Genet. 91:137–141.

Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. *In* Cryopreservation of Tropical Germplasm. Current Research Progress and Application. Eds. F. Engelmann and H. Takagi. JIRCAS, Rome, pp 8–20

Galbraith, D.W., G. Lambert, J. Macas and J. Doležel. 2002. Analysis of nuclear DNA content and ploidy in higher plants. *In* Current Protocols in Cytometry. Eds. J. Robinson, Z. Darzynkiewicz, P. Dean, A. Hibbs, A. Orfão, P. Rabinovitch and L. Wheeless. Wiley, New York.

Galbraith, D.W., K.R. Harkins, J.M. Maddox, N.M. Ayres, D.P. Sharma and E. Firoozabady. 1983. Rapid flow cytometric analysis of the cell-cycle in intact plant tissues. Science 220:1049–1051.

Gonzalez-Arnao, M.T., F. Engelmann, C.U. Villavicencio, M. Morenza and A. Rios. 2000. Cryopreservation of citrus apices using the encapsulation-dehydration technique. *In*

Cryopreservation of Tropical Germplasm. Current Research Progress and Application. Eds. F. Engelmann and H. Takagi. JIRCAS, Rome, pp 217–221.

Gonzalez-Arnao, M.T., J. Juarez, C. Ortega, L. Navarro and N. Duran-Vila. 2003. Cryopreservation of ovules and somatic embryos of citrus using the encapsulation-dehydration technique. Cryoletters 24:85–94.

Gonzalez-Benito, M.E., G. Garcia-Martin and J.A. Manzanera. 2002. Shoot development in *Quercus suber* L. somatic embryos. In Vitro Cell. Develop. Biol.-Plant 38:477–480.

Grout, B.W.W. 1986. Embryo culture and cryopreservation for the conservation of genetic resources of species with recalcitrant seed. *In* Plant tissue culture and its agricultural applications. Eds. L.A. Withers and P.G. Alderson. Butterworth, London, pp 303–309.

GROUT, B.W.W. 1995. Introduction to the in vitro preservation of plant cells, tissues and organs. *In* Genetic Preservation of Plant Cells In Vitro. Eds. Springer-Verlag. Heidelberg, pp 1–20.

Hao, Y.J., C.X. You and X.X. Deng 2002*a*. Effects of cryopreservation on developmental competency, cytological and molecular stability of citrus callus. Cryoletters 23:27–35.

Hao, Y.J., C.X. You and X.X. Deng 2002*b*. Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation. Cryoletters 23:37–46.

Hirata, K., M. Phunchindawan, J. Tukamoto, S. Goda and K. Miyamoto. 1996. Cryopreservation of microalgae using encapsulation-dehydration. Cryoletters 17:321–328.

Hornero, J., I. Martinez, C. Celestino, F.J. Gallego, V. Torres and M. Toribio. 2001. Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. International J. Plant Sci. 162:827–833.

Ishida, T.A., K. Hattori, H. Sato and M.T. Kimura. 2003. Differentiation and hybridization between *Quercus crispula* and *Q.dentata* (Fagaceae): Insights from morphological traits, amplified fragment length polymorphism markers, and leafminer composition. Am. J. Bot. 90:769–776.

Kampfer, S., C. Lexer, J. Glossl and H. Steinkellner. 1998. Characterization of (GA)(n) microsatellite loci from *Quercus robur*. Hereditas 129:183–186.

Leal, F., J. Loureiro, E. Rodriguez, M.S. Pais, C. Santos and O. Pinto-Carnide. 2006. Nuclear DNA content of *Vitis vinifera* cultivars and ploidy level analyses of somatic embryo-derived plants obtained from anther culture. Plant Cell Rep. 25:978–985.

Liu, Y., X. Wang and L. Liu. 2004. Analysis of genetic variation in surviving apple shoots following cryopreservation by vitrification. Plant Sci. 166:677–685.

Liu, Y.G., L.X. Liu, L. Wang and A.Y. Gao. 2008. Determination of genetic stability in surviving apple shoots following cryopreservation by vitrification. Cryoletters 29:7–14.

Lopes, T., G. Pinto, J. Loureiro, A. Costa and C. Santos. 2006. Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. Tree Physiol. 26:1145–1152.

Loureiro, J., A. Capelo, G. Brito, E. Rodriguez, S. Silva, G. Pinto and C. Santos 2007. Micropropagation of *Juniperus phoenicea* from adult plant explants and analysis of ploidy stability using flow cytometry. Biol. Plant. 51:7–14.

Loureiro, J., E. Rodriguez, J. Dolezel and C. Santos. 2006. Comparison of four nuclear isolation buffers for plant DNA flow cytometry. Ann. Bot. 98:679–689.

Loureiro, J., E. Rodriguez, J. Dolezel and C. Santos. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: A test with 37 species. Ann. Bot. 100:875–888.

Loureiro, J., G. Pinto, T. Lopes, J. Dolezel and C. Santos. 2005. Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry. Planta 221:815–822.

Malik, S.K., R. Chaudhury, O.P. Dhariwal and R.K. Kalia. 2006. Collection and characterization of *Citrus indica* Tanaka and *C-macroptera* Montr.: wild endangered species of northeastern India. Genetic Res. Crop Evol. 53:1485–1493.

Martinez, M.T., A. Ballester and A.M. Vieitez. 2003. Cryopreservation of embryogenic cultures of *Quercus robur* using desiccation and vitrification procedures. Cryobiology. 46:182–189.

Muller, J., J.G. Day, K. Harding, D. Hepperle, M. Lorenz and T. Friedl. 2007. Assessing genetic stability of a range of terrestrial microalgae after cryopreservation using amplified fragment length polymorphism (AFLP). Am. J. Bot. 94:799–808.

Murashige, T. and F. Skoog. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol. Plant. 15:473–497.

Niino, T. and A. Sakai. 1992. Cryopreservation of Alginate-Coated Invitro-Grown Shoot Tips of Apple, Pear and Mulberry. Plant Sci. 87:199–206.

Panis, B. and M. Lambardi. 2005. Status of cryopreservation technologies in plants (crops and forest trees). *In* The role of biotechnology for the characterisation and conservation of crop, forest, animal and fishery genetic resources in developing countries. Eds. FAO. Turin, Italy, pp 43–54.

Pâques, M., V. Monod, M. Poissonnier and J. Dereuddre. 2002. Cryopreservation of *Eucalyptus* sp. shoot tips by the encapsulation-dehydration procedure. *In* Biotechnology in Agriculture and Forestry 50. Eds. L.E. Towill and Y.P.S. Bajaj Springer-Verlag, Berlin. pp 234–245.

Park, Y.S. 2002. Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann. For. Sci. 59:651–656.

Pinto, G., H. Valentim, A. Costa, S. Castro and C. Santos. 2002. Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. tree. In Vitro Cellular & Developmental Biology-Plant. 38:569–572.

Sakai, A. 2004. Plant cryopreservation. *In* Life in the Frozen State. Eds. B.J. Fuller, N. Lane and E.E. Benson. CRC Press, pp 329–345

Santos, C., J. Loureiro, T. Lopes and G. Pinto. 2007. Genetic fidelity of in vitro propagated cork oak (*Quercus suber* L). *In* Protocols for Micropropagation of Woody Trees and Fruits. Eds. S.M. Jain and H. Häggman. Springer-Verlag, Berlin, pp 67–84.

Scocchi, A., S. Vila, L. Mroginski and F. Engelmann. 2007. Cryopreservation of somatic embryos of paradise tree (*Melia azedarach* L.). Cryoletters 28:281–290.

Shibli, R.A., D.M. Haagenson, S.M. Cunningham, W.K. Berg and J.J. Volenec. 2001. Cryopreservation of alfalfa (*Medicago sativa* L.) cells by encapsulation-dehydration. Plant Cell Rep. 20:445–450.

Steinkellner, H., S. Fluch, E. Turetschek, C. Lexer, R. Streiff, A. Kremer, K. Burg and J. Glossl. 1997. Identification and characterization of (GA/CT)(n)-microsatellite loci from *Quercus petraea*. Plant Mol. Biol. 33:1093–1096.

Takagi, H. 2000. Recent developments in cryopreservation of shoot apices of tropical species. *In* Cryopreservation of Tropical Germplasm. Current Research Progress and Application. Eds. F. Engelmann and H. Takagi. IPGRI, Rome, pp 178–193.

Tessereau, H., B. Florin, M.C. Meschine, C. Thierry and V. Petiard. 1994. Cryopreservation of somatic embryos—a tool for germplasm storage and commercial delivery of selected plants. Ann. Bot. 74:547–555.

Valladares, S., M. Toribio, C. Celestino and A.M. Vieitez. 2004. Cryopreservation of embryogenic cultures from mature *Quercus suber* trees using vitrification. Cryoletters 25:177–186.

Vendrame, W.A., G. Kochert and H.Y. Wetzstein 1999. AFLP analysis of variation in pecan somatic embryos. Plant Cell Rep. 18:853–857.

Vendrame, W., C. Holliday, P. Montello, D. Smith and S. Merkle 2001. Cryopreservation of yellow-poplar and sweetgum embryogenic cultures. New For. 21:283–292.

Verleysen, H., E. Van Bockstaele and P. Debergh. 2005b. An encapsulation-dehydration protocol for cryopreservation of the azalea cultivar 'Nordlicht' (*Rhododendron simsii* Planch.). Sci Hortic. 106:402–414.

Verleysen, H., G. Samyn, E. Van Bockstaele and P. Debergh. 2004. Evaluation of analytical techniques to predict viability after cryopreservation. Plant Cell Tissue Organ Cult. 77:11–21.

Verleysen, H., P. Fernandes, I.S. Pinto, E. Van Bockstaele and P. Debergh. 2005. Cryopreservation of *Robinia pseudoacacia*. Plant Cell Tissue Organ Cult. 81:193–202.

Volk, G.M., J.L. Harris and K.E. Rotindo. 2006. Survival of mint shoot tips after exposure to cryoprotectant solution components. Cryobiology 52:305–308.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Vandelee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP - A new technique for DNA-fingerprinting. Nucleic Acids Res. 23:4407–4414.

Wilhelm, E., K. Hristoforoglu, S. Fluch and K. Burg 2005. Detection of microsatellite instability during somatic embryogenesis of oak (*Quercus robur* L.). Plant Cell Rep. 23:790–795.

Wilkinson, T.I.M., A. Wetten, C. Prychid and M.F. Fay. 2003. Suitability of Cryopreservation for the Long-term Storage of Rare and Endangered Plant Species: a Case History for *Cosmos atrosanguineus*. Ann Bot. 91:65–74.

Chapter VI

Screening of ten selected cork oak genotypes through somatic embryogenesis

Chapter section submitted as original article in SCI journal:

Fernandes P, Pinto G, Roldan-Ruiz I, De Loose M, Santos C (2011). Screening of ten selected cork oak genotypes through somatic embryogenesis *Tree Physiology* (submitted).

Abstract

Ten trees hundred-year old were chosen from Companhia das Lezírias, Portugal, based on its cork production ability as it follows: good cork producers CL2, CL12, CL22, CL36 and CL45; bad cork producers CL5, CL8, CL11, CL15 and CL38. Somatic embryogenesis was induced in MS culture media supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid and 9.0 μM zeatin. Somatic globular embryo clusters were isolated and transferred to MS culture medium complemented with 5% (w/v) PEG 6000.After maturation during 4 weeks cotyledonar embryos were transferred to basal MS media and submitted to chilling (4º C) during 8 weeks. For conversion, WPM media supplemented with 0.5 $mg.L^{-1}$ BAP and 0.1 $mg.L^{-1}$ 1-naphthaleneacetic acid (NAA) was used. After conversion was achieved plantlets were acclimatized. After induction conditions, all genotypes responded embryogenically with exception of CL5 and CL38. Genotype CL11 showed the highest mass increment while CL15 showed the highest conversion percentage. CL12 never responded to conversion treatments.

DNA-marker analyses (AFLP) revealed no significant differences between control and cryopreserved samples at the DNA-sequence level.

The presented protocol proved to be stable for 7 out of ten genotypes achieving conversion with no significant genetic variability, proving its ability to be use in large scale production programs.

Keywords: AFLP, cork oak, genotype, somatic embryogenesis.

Introduction

Cork oak (*Quercus suber* L.) is a species of major importance throughout the world due to cork transformation industry and plays an important role in Southern European ecosystems (Pinto *et al.*, 2002, Loureiro *et al.*, 2005). However, as a consequence of the oak population aging and susceptibility to environmental factors (e.g. fungus-host interactions, forest fires) a large percentage of cork oak populations are declining (e.g. FAO 1996). Moreover, classical cork oak breeding programs are conditioned by vegetative propagation limitations and by low rates of seed conservation, while in vitro propagation is a promising tool and may provide an opportunity to develop "clonal varieties" of elite clones with genetic superiority in the field (Park, 2002)

It is well known that in vitro culture can induce somaclonal variation (by e.g. mutation and/or epigenetic changes) (e.g. Kaeppler *et al.*, 2000; Berlyn *et al.*, 1986; Larkin and Scowcroft, 1981), which may hamper the implementation of clonal forestry programs or, on the other hand, may provide interesting mutants. From all the in vitro techniques used, somatic embryogenesis (SE) is the most promising method for clonal mass propagation of forest species (Merkle, 1995), mostly because both root and shoot meristems are present (Kim, 2000).

In Q. suber, SE was achieved from leaves of seedlings (Fernández-Guijarro *et al.*, 1995), nodal segments (Elmaataoui and Espagnac, 1987), zygotic embryos (Bueno *et al.*, 1992; Manzanera *et al.*, 1993), and from leaf explants of juvenile (Hernández *et al.*, 1999; Hornero *et al.*, 2001; Toribio *et al.*, 1998) and adult plants (Pinto *et al.*, 2001; Pinto *et al.*, 2002). However, for the inclusion of a SE-protocol in breeding programs, plant quality (e.g. genetic variability) and performance must be assessed.

Using Q. suber somatic embryos from several embryogenic lines obtained from zygotic embryos, no somaclonal variation has been detected by Random Amplified Polymorphic DNA (RAPD) analyses (Gallego, 1997). This result was later confirmed for several embryogenic lines by Amplified Fragment Length Polymorphisms (AFLP) markers (Hornero *et al.*, 2001). However, when using embryogenic lines from mature explants, AFLP analyses detected somaclonal variation in one genotype. Using the SE protocol developed by Pinto *et al*, (2002) for this species, Loureiro *et al.* (2005) found no ploidy or DNA content variations among somatic embryos derived from mature cork oaks. Using SSR markers, Lopes *et al.* (2006) found only one mutation in one embryogenic line derived from an adult tree, while other lines also derived from adult or young genotypes remained stable. These data suggest that

the process, age of explant and/or genotype influence the genetic stability, which leads to the necessity of a tight genetic control throughout the SE process.

AFLP, a very sensitive multi-locus fingerprinting technique (Wilkinson *et al.,* 2003), was already used to assess genetic diversity in the *Quercus* genus (Hornero *et al.,* 2001, Coart *et al.*, 2002, Ishida *et al.*, 2003, Fernandes *et al.,* 2008) proving its reliability. It is an easy-to-use technique that allows a quick and wide screening of the genome and can be easily used at different stages of the SE process since the field plant, trough the somatic embryos until the emblings.

In this experiment SE competence and success was evaluated in order to establish a reliable breeding protocol based on field elite lines. More, genetic stability was assessed by AFLP along the process to ensure that no variations are induced during the SE process until the embling.

Material and Methods

Plant material

Ten trees hundred-year old were chosen from Companhia das Lezírias, Portugal, based on its cork production (data from C. Lezírias) ability as it follows: good cork producers CL2, CL12, CL22, CL36 and CL45; bad cork producers CL5, CL8, CL11, CL15 and CL38. Branch segments, collected during December 2006, devoid of lateral branches and leaves, up to 4 cm diameter and 15 cm in length were forced to sprout in a greenhouse at 24±2ºC and 80–95% relative humidity. They were sprayed weekly with a solution of Benomil (500mg/L; 50%) + Carbendazin (80 mg/L; 8%) + Captan (200mg/L; 40%). Expanding leaves were excised and used without any further wounding as initial explants. They were surface sterilized by vigorous shaking in 70% EtOH for 30 s, followed by immersion in a 10% NaClO plus two drops of Tween 20 for 2 min, followed by three rinses in sterile distilled water. Five leaves were placed with the abaxial surface to the culture medium, in disposable Petri dishes filled with 10 ml medium and sealed with Parafilm.

Media composition

The induction of somatic embryogenesis was performed following the method described in Pinto *et al.* (2002). Induction medium (S6) during this phase consisted of MS (Murashige and Skoog, 1962) medium with 30 g/l sucrose, 2.5 g/l Gelrite® and supplemented with 1 mg/L 2,4–dichlorophenoxyacetic acid (2,4-D) and 2 mg/L zeatin. The medium for expression (MSWH) of somatic embryogenesis and recurrent proliferation had the same components,

but lacked PGRs (Pinto *et al.,* 2002), while maturation medium was complemented with 5% (w/v) polyethylene glycol 6000 (PEG 6000). For chilling procedures, expression medium was used. For conversion, WPM media (woody plant medium, Lloyd and McCown, 1980) supplemented with 0.5 $mg.L^{-1}$ BAP and 0.1 $mg.L^{-1}$ 1-naphthaleneacetic acid (NAA) was used. All media were adjusted to pH 5.8 prior to autoclaving at 121ºC for 20 min.

Experimental procedure and culture conditions

Surface-sterilized leaves were cultured on the induction medium in the dark at 25±2ºC until somatic embryogenic callus are formed. Induction rates and genotype effect was evaluated. These were then transferred to the expression and proliferation medium in a growth chamber with a 16-h photoperiod provided by mixed SYLVANIA GRO-LUX and PHILIPS cool-white fluorescent tubes (90–120 $\mu mol.m^2.s^{-1}$), at 25±2ºC and subcultured monthly. Afterwards, immature white-opaque dicotyledonary somatic embryos were isolated and transferred to maturation medium and maturated during 30 days under the same light/temperature conditions as above. Afterwards, these embryos were transferred to chilling medium and kept at 4±1ºC in the dark during two months. After this period they were transferred to both conversion media until germination occurs, with monthly transfers to fresh medium. Emblings with at least 1cm of root and shoot were then transferred to forest containers filled with substrate (pine bark:peat:perlite, 1:2:1, v/v/v). These potted plantlets were kept inside glass beakers. After 1 month, the beakers were opened for 1 h/day for 1 month. Next, the plants were moved a growth chamber under 90–120 $\mu mol.m^2.s^{-1}$, at 25±2ºC and constant high humidity (HR=90%). During two weeks HR was decreased until environment values and plants were re-potted and moved to the greenhouse.

DNA extraction

Sprouted leaves from field collected branches, somatic embryogenic clusters and embling leaves form all ten genotypes were immersed in liquid nitrogen and lyophilized for 48 h. Dried material (20 mg) was ground and DNA was isolated according to the Qiagen extraction kit procedure. This method yielded up to 20 µg of genomic DNA per extraction. DNA concentrations were determined using Nanodrop 1000 Spectrophotometer (Thermo Scientific).

AFLP reactions

AFLP were performed according to Vos *et al.* (1995). Genomic DNA (288 ng) was digested for 2 h at 37 °C in a final volume of 25 µl containing 50 mM of MgAc, 250 mM of KAc, 50 mM Tris-HCl pH 7.5, 2.5 U of *Eco*RI (Invitrogen) and 2.5 U of *Mse*I (Invitrogen). Two adaptors, one for the *Eco*RI ends and one for the *Mse*I ends, designed to avoid the reconstruction of the restriction sites, were ligated to the restriction fragments by adding 24 µl of a mix containing 5 pmol of *Eco*RI adaptor, 50 pmol of *Mse*I adaptor, 10 mM of ATP, 1M of Tris-HCl, 1M of MgAc, 2M of KAc and 1 U of T4 DNA ligase (Invitrogen). The ligation mixture was incubated for 2 h at 37 °C. A pre-amplification step was performed with primers complementary to the *Eco*RI and *Mse*I adaptors with an additional selective 3′ nucleotide. The PCR reactions were performed in a 50 µl volume of 10X PCR Buffer (Applied Biosystems), 5 mM of each dNTP, 25 ng of each primer (Invitrogen), 1.25 U of Taq DNA polymerase (Applied Biosystems) and 5 µl of the restriction products. The PCR amplifications were carried out in a Perkin Elmer Geneamp PCR system 9600 using 25 cycles, each cycle consisting of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. For the selective amplification, 4 primer sets with three selective nucleotides were selected based on Fernandes *et al.* (2008) work: *Eco*RI-ACG/*Mse*I-CAC, *Eco*RI-AGC/*Mse*I-CAA *Eco*RI-ACC/*Mse*I-CAT *Eco*RI-ACG/*Mse*I-CTC. The *Eco*RI primer was labelled with one of the three fluorescent dyes: HEX, FAM, NED. The PCR amplification mixture was composed of 3 µl of diluted pre-amplification product (1/10 of their initial concentration), 1 µl of *Mse*I primer at 5 µM, 1 µl of *Eco*RI primer at 1 µM, 0.6 U of Taq DNA polymerase (Applied Biosystems), 2 µl of 10X PCR Buffer (Applied Biosystems) and 0.2 µl of dNTPs (20-mM each Invitrogen). The selective amplification was carried with the following parameters: 1 cycle of 2 min at 94 °C, 30 s at 65 °C, 2 min at 72 °C, followed by eight cycles in which the annealing temperature decreases 1 °C per cycle, followed by 23 cycles of 1 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C. Samples were analyzed using an ABI Prism 3130 DNA sequencer (Applied Biosystems) using HEX, FAM, NED multiplexes. GeneScan Analysis Software 3.1.2 (Applied Biosystems) was used to translate the information collected by the ABI3130 into fragment sizing information. In all cases a peak amplitude threshold of 50 was set for the analysis. The GeneScan files were scored and recorded with GeneMapper 3.7 (Applied Biosystems), and a binary matrix was generated using Microsoft Excel. AFLP bands were scored within the size range of 50–2000 bp.

Data analysis

The number of leaves initially introduced in culture per treatment was variable, depending on the availability of branches. Data on the number of remaining leaves after contamination and the number of leaves showing somatic embryo formation were recorded up 8 weeks in induction. For proliferation rates assessment, two replicates of five clusters each per genotype were evaluated based on number of cotiledonary staged somatic embryos and weight variation after one month. The number of maturated selected embryos was variable according to its availability. After chilling and conversion/germination treatments, number of converted/germinated embryos was recorded. Germinated plants were recorded and, after acclimatization, number of *ex vitro* cultured plantlets showing active growth after 2 months was registered. To evaluate statistical differences in all treatments, an ANOVA was used, in combination with the post-hoc Duncan, Holm-Sidak or Tukey-Krammer multiple comparison tests. AFLP-marker profiles were compared using the Simple Matching coefficient. The software package SPSS for Windows (version 16.0, SPSS Inc.) was used for all calculations.

Results

Sprouting efficiency

Sprouting of epicormic shoots from field-collected segments of branches was abundant and easy to obtain. After 1 week under high humidity, newly formed buds appeared, emerging through the bark, though no size-collectable were yet present. Two weeks later, the first expanding leaves could be harvested (Figure 16). Leaves and epicormic shoots continued sprouting and growing for at least five weeks before wilting took place.

Nevertheless, different sprouting efficiencies were observed among genotypes. After 3 weeks under sprouting conditions, it was clear that some genotypes presented higher leave production per branch segment (Figure 17). Since branches were collected from identical tree areas and were selected based on their similarity, this indicates a clear genotype effect.

Figure 16: Epicormic shoots sprouted in fragments of branches from a hundred-year-old cork oak tree, bearing expanded leaves. Scale bar: 1 cm.

Genotypes CL5, CL15 and CL45 presented approximately two fold production of leaves per branch compared to all other genotypes (P<0.001). These results were also observed after 4 weeks (Figure 17). Despite the previous characterization of the field trees in either good or bad cork producers, no correlation was found with its sprouting ability.

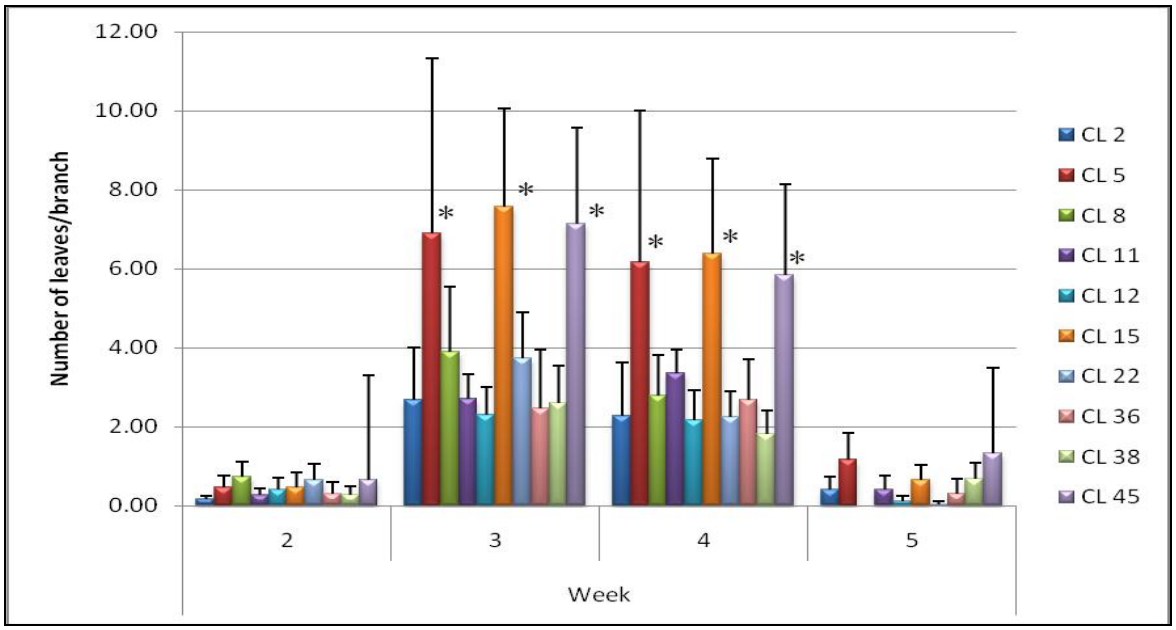

Figure 17: Sprouting efficiency measured as number of leaves per branch segment. *P<0.001 Holm-Sidak: Bars represent standard error.

Somatic embryogenesis induction

Somatic embryogenesis induction was evaluated at two different stages: after the 8 weeks in induction conditions and after 8 weeks of removal from induction conditions. The presence of both embryogenic and non-embryogenic calli was assessed according to the description of Pinto *et al.* (2002).

After 8 weeks under dark on S6 culture medium a total average of 28% of all explants produced some type of calli, except genotype CL5. The total average of explants showing embryogenic tissue was about 16%, while non-embryogenic tissue was found in 39% of the explants.

Figure 18: **Somatic** embryos emergence from a sprouted leave in S6 culture medium

Nevertheless, almost all explants presenting embryogenic calli also produced non-embryogenic, while the opposite was rarely verified (Figure 18).

At this stage only genotype CL5 presented no formation of any calli while CL38 only resulted in about to 2% of non-embryogenic calli. On the other hand, genotypes CL12 and CL15 reached more than 56% of embryogenic calli production. After 8 weeks in induction media, non-embryogenic calli production was always higher than embryogenic calli response, with exception of CL12 (Table 19), reaching in some cases more than 70% (CL45).

Following transferring all explants to PGR-free media (MSWH), evaluation was made after 8 weeks. A clear increase in explants producing either or both types of calli was obvious (Table 20). Embryogenic calli production/proliferation reached a total of 29% (an increase of 12%), while non-embryogenic calli presence increased 5%. Once again there are genotypes responding significantly better. This is the case of CL2, CL8, CL11, CL12, CL15 and CL45. Genotypes CL22 and CL38, despite some increase in calli production, did not proliferate to recurrent embryogenesis and were lost.

Table 20: Percentage of leaves producing either embryogenic or non-embryogenic calli after 8 weeks in S6 culture media. Means±SE are from 2 to 31 replicates (genotypes CL 2 and CL 22). Means within the same column followed by the same letter are not significantly different at P<0.05 (Tukey's multiple comparison test).

Genotype	Embryogenic calli	Non-Embryogenic calli
CL 2	30.00±7.07 **abc**	50.00±35.36 **bcd**
CL 5	0.00±0.00 **a**	0.00±0.00 **a**
CL 8	26.00±5.12 **ab**	60.00±7.75 **cd**
CL 11	21.90±4.24 **a**	62.86±6.36 **cd**
CL 12	57.50±10.27 **c**	42.50±8.24 **abcd**
CL 15	56.36±7.64 **bc**	69.09±10.08 **d**
CL 22	1.94±1.06 **a**	10.97±3.00 **ab**
CL 36	0.67±0.66 **a**	23.33±3.90 **abc**
CL 38	0±0.00 **a**	1.67±1.13 **a**
CL 45	23.33±4.63 **a**	72.00±4.38 **d**
$\mathbf{F_{(6,169)}}$	12.882 P<0.05	23.867 P<0.05

Multiplication

In order to evaluate the differences between assessments, immediately following induction and after 8 weeks in MSWH, proliferation and multiplication of embryogenic calli were studied. All 7 genotypes which survived and produced embryogenic calli were evaluated.

After one month in proliferation media, individual calli weight and number of globular and cotyledonary-staged embryos, was recorded (Figures 19A, B and C). Clusters mass variation was very similar for all genotypes. Nevertheless, CL 11 presented a higher mass production, while CL 2 had the lowest mass increment (Figure 19A).

Table 21: Percentage of leaves producing either embryogenic or non-embryogenic calli after 8 weeks in S6 culture media, following 8 weeks in MSWH culture media. Means±SE are from 2 to 31 replicates (genotypes CL 2 and CL 22). Means within the same column followed by the same letter are not significantly different at P<0.05 (Tukey's multiple comparison test).

Genotype	Embryogenic calli	Non-Embryogenic calli
CL 2	40.00±14.14 **abcde**	60.00±28.28 **bc**
CL 5	0.00±0.00 **a**	0.00±0.00 **a**
CL 8	46.00±6.80 **abcde**	66.00±7.76 **c**
CL 11	50.48±7.57 **cde**	69.52±5.32 **c**
CL 12	57.50±12.47 **e**	50.00±11.73 **abc**
CL 15	54.54±10.63 **de**	69.09±11.31 **c**
CL 22	6.45±2.48 **abc**	10.32±3.14 **ab**
CL 36	8.00±3.07 **abcd**	42.67±7.04 **abc**
CL 38	3.33±1.52 **ab**	8.33±2.01 **a**
CL 45	48.67±6.65 **bcde**	69.33±4.97 **c**
$\mathbf{F_{(6,169)}}$	13.275 P<0.05	15.785 P<0.05

This results when crossed with the number of globular and cotyledonar embryos show that increase in CL 11 is due to a significant increase in the number of globular embryos instead their evolution to cotyledonar. On the other hand is CL 36 which has its mass increase related to the evolution of their initial globular embryos to the cotyledonary stage. This behaviour was also verified in CL 8 (Figures 19B and C). CL 45 and CL 15 presented no significant changes in mass increment, globular embryos appearance and cotyledonar evolution (Figures 19A, B and C).

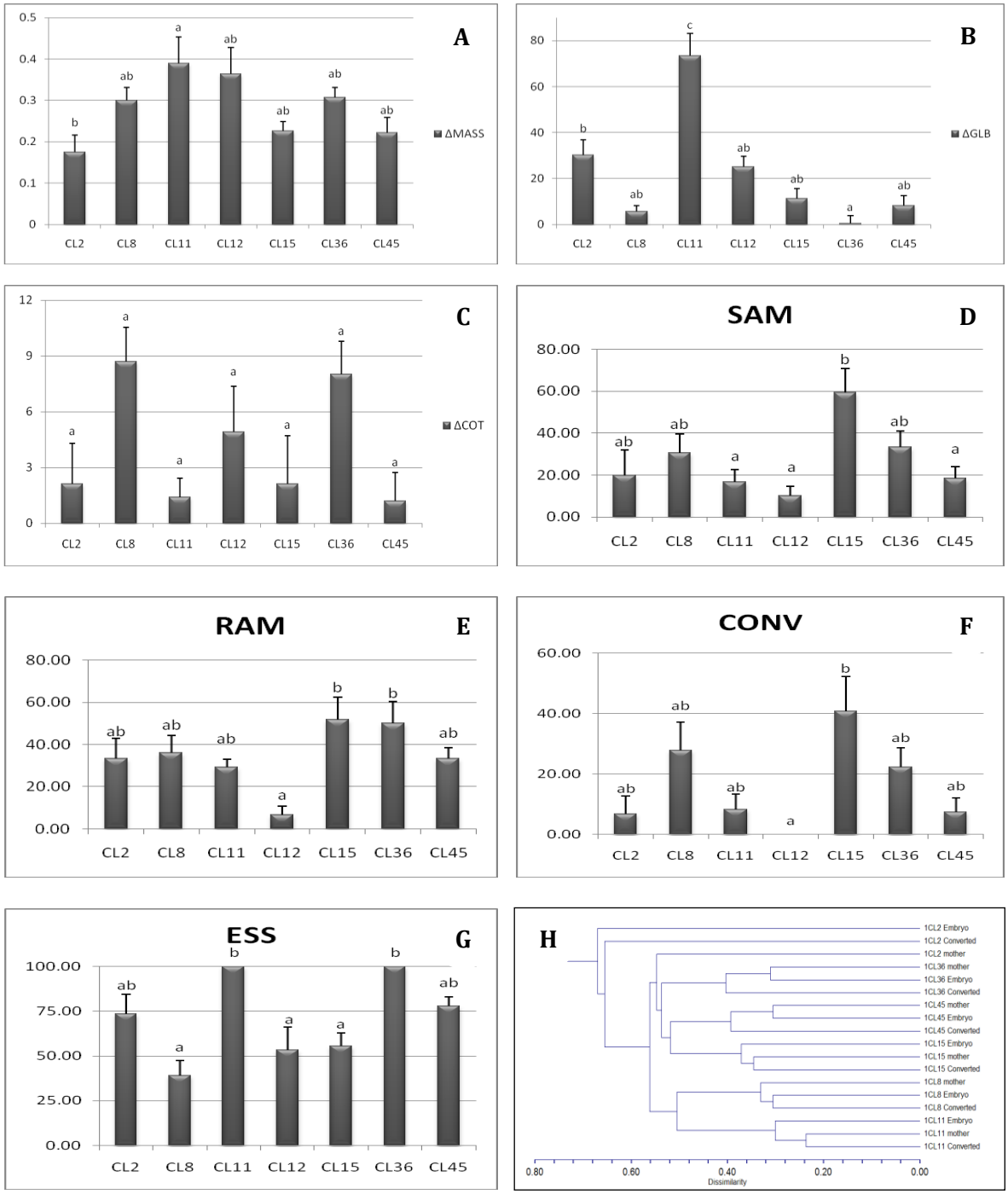

Figure 19: A: Weight variation (Δg) after one month in proliferation medium. $F_{(6,63)}$=3.017 P<0.05. **B:** Number of globular-staged somatic embryos (ΔGLB) after one month in proliferation medium. $F_{(6,63)}$=18.587 P<0.05. **C:** Number of cotyledonar-staged somatic embryos (ΔCOT) after one month in proliferation $F_{(6,63)}$=2.300 P<0.05. **D:** After two months in conversion culture medium. $F_{(6,58)}$=3.509 P<0.05. **E:** After two months in conversion culture medium. $F_{(6,58)}$=3.479 P<0.05. **F:** Conversion after two months in conversion culture medium. $F_{(6,58)}$=3.391 P<0.05. **G:** Secondary somatic embryogenesis after two months in conversion culture medium. $F_{(6,58)}$=6.886 P<0.05. Tukey post-hoc was performed for statistical analysis. Bars represent standard error. **H:** cluster hierarchy analysis resultant from a simple matching similarity matrix.

Conversion

Mature isolated embryos were transferred to MSWH medium and after two months chilling, were moved into WPM and MS media supplemented with NAA and BAP. Evaluation of conversion was made monthly and the number of embryos presenting development of shoot apical meristems (SAM), root apical meristems (RAM), or both (conversion) was recorded, as well as secondary somatic embryogenesis (SSE). For all evaluated parameters it was clear the genotype effect. CL12 always presented low SAM, RAM and no conversion at all. Root emergence was usually more frequent than shoot appearance which leaded to low synchronization and consequent low conversion (Figures 19D, E and F). Nevertheless CL15, CL8 and CL36 presented conversion as high as 40%. However, secondary somatic embryogenesis was always present, sometimes in all explants (Figure G) causing usually a "downgrade" of plant material to repetitive somatic embryogenesis state.

AFLP assay

The six genotypes which regenerated via somatic embryogenesis (CL2, CL8, CL11, CL15, CL36 and CL45) were analyzed at three different stages: mother plant, somatic embryo and converted plant. All samples revealed a total of 301 AFLP markers. Four different selective primer pairs were used and each plant produced individual markers in the range of 115 (for plant CL45 mother plant) to 146 (for plant CL2 converted plant).

The average number of fragments per primer pair and per plant was 131.4. All regenerated plants analyzed shared 25 (8.3%) non-polymorphic markers. There were 82 (27.2%) singletons or AFLP fragments either present or absent in just one plant: 59 (19.6%) were amplification singletons and 23 (7.6%) were non-amplified singletons.

A cluster hierarchy analysis (Figure 19H) to all samples showed a clear distribution accordingly to the genotype. It is clear the proximity between the different somatic embryogenic stages (Table 21) of a same genotype. There are two clear main groups enclosing CL15, CL36, CL45 and CL8 and CL11. CL2 presented itself very far from all other genotypes, especially for somatic embryos which are completely aside all other samples. Despite these grouping similarities no relations were found with cork producing neither ability nor somatic embryogenesis/conversion response. Nevertheless this results show stability during the entire somatic embryogenesis process.

This study allowed us to enhance the previous protocol for cork oak somatic embryogenesis cited by Lopes *et al.* (2006), improving particularly maturation and conversion and adding an efficient cryopreservation step (Fernandes *et al.,* 2008) (Figure 20).

Table 22: Summary of AFLP fragments variation in the six genotypes analyzed.

Primer pair	Number of AFLP markers	Number of polymorphic markers					
		Genotype					
*Eco*RI+3/*Mse*I+3		CL2	CL8	CL11	CL15	CL36	CL45
ACG/CAC	79	38	10	7	21	17	15
AGC/CAA	66	34	13	6	12	12	14
ACC/CAT	82	64	11	9	12	17	9
ACG/CTC	74	40	8	10	11	12	18

Discussion

This study intended to follow ten cork oak selected genotypes based on their cork production characteristics, form donor field plants until new plants were regenerated through somatic embryogenesis. They were selected based on information provided by ISA, resultant from several cork physical and chemical properties evaluations.

Sprouting efficiency

Using this procedure we manage to have a controlled supply of leaves that could be readily used for induction of somatic embryogenesis. One of the theoretical advantages of this controlled material compared to field collected material is that aseptic cultures can be obtained more easily, since leaves are sprouted in a contamination free environment and fungicides/bactericides might be used. Nevertheles, losses of explants due to contamination were extremely variable and no correlation was found with genotypes (data not shown). Same results were reported by Hernández *et al.* (2003a, 2003b). Regarding sprouting efficiency itself, genotypes CL5, CL15 and CL45 presented approximately twice more leaves than other genotypes. Since sprouting conditions were the same for all explants, and branches were identical and collected at the same time, we can only conclude a genotype effect for vegetative propagation. Hernandez *et al.* (2003a, 2003b) reports that this variability might also be due to collection dates or mother plant location. However, in our case this is not a variable, since branches were collected at same time and place being genotype the only answer.

Induction of somatic embryogenesis

It can be confirmed, following the results of previous reports (Fernández-Guijarro 1997; Toribio *et al.* 2000; Hernández *et al.* 2001) and data presented here, that the induction of somatic embryogenesis from leaves of mature trees of cork oak is a reliable trait.

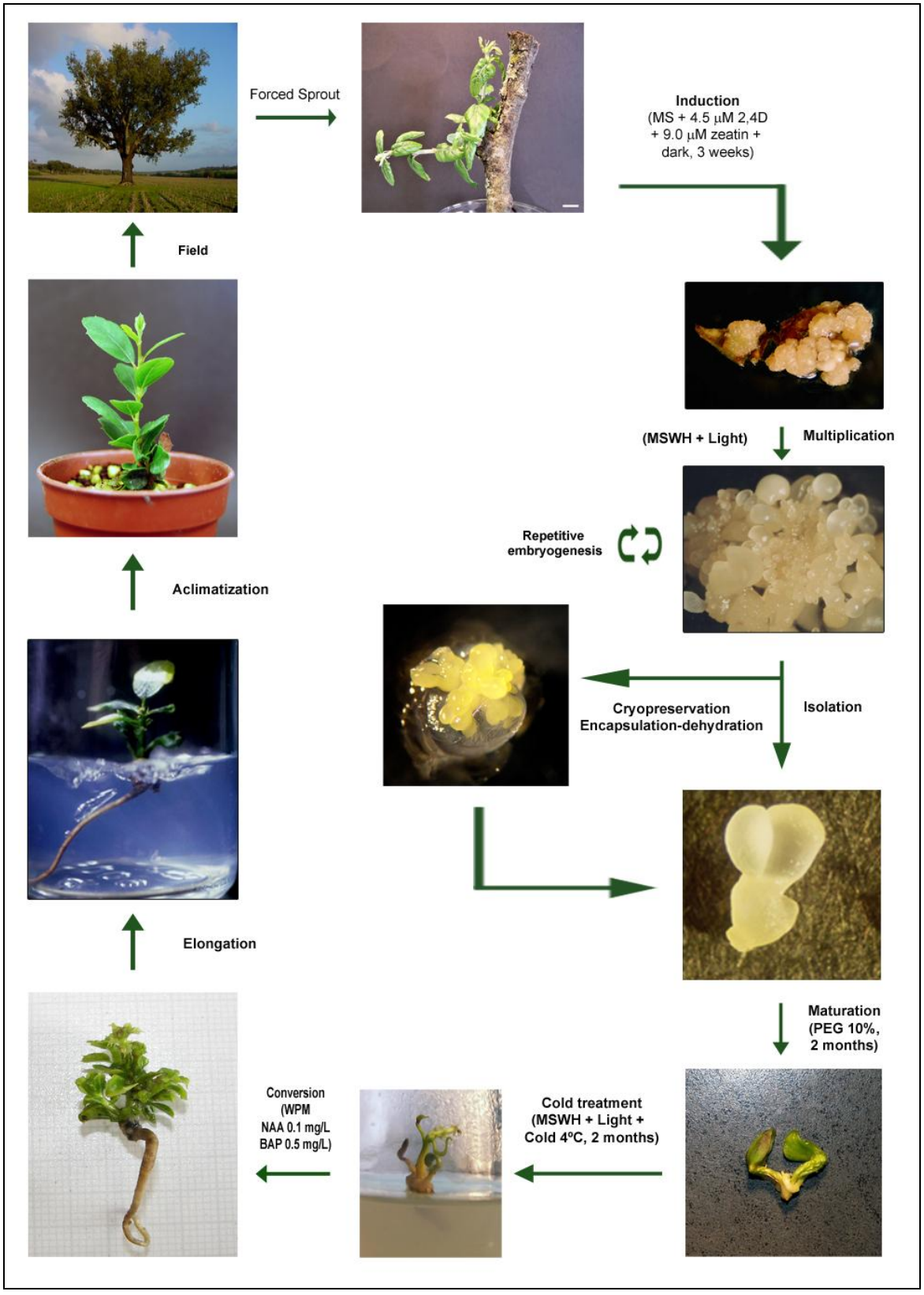

Figure 20: Enhanced protocol for cork oak somatic embryogenesis from mature plants selected from the field for their cork oak production characteristics.

The method reported by Pinto *et al.* (2002) once again proved to be a reliable and cost sensitive one step method when compared with two step methods (Hernandez *et al.*, 2003a). The use of a single induction culture media followed by complete PGR removal after 8 weeks, resulted in 29% of embryogenic calli production. At this point, this is one of the highest values ever reached. However, despite all above the proliferation of non embryogenic calli was almost always present, though it was lowered when PGRs were removed. This indicates that used hormones (2,4-d and zeatin) might have an excessively proliferation effect on exposed cells. Fernández-Guijarro *et al.* (1995) report the use of NAA and BAP as essential to induce somatic embryogenesis in cork oak leaves. It is widely accepted the use of PGRs for SE induction, but little is known about its long-term effect.

The influence of genotype was clear at both PGR and PGR-free media. Genotype CL5 never had any kind of response, embryogenic or non-embryogenic. Moreover, genotypes CL22 and CL38 produced calli, but not enough to proliferate. Genotype effect on morphogenic processes is well documented, including the genetic control of somatic embryogenesis induced in zygotic embryos (Cheliak and Klimaszewska 1991; Park *et al.,* 1993; Kim *et al.,* 1997; Fernández-Guijarro, 1997) or in non-embryonic tissues (Barro *et al.*, 1999). A genotypic effect on the induction of embryogenesis in leaves from Quercus robur seedlings has also been suggested (Cuenca *et al.,* 1999).

Although we recorded a large variation in the frequency of induction among genotypes, embryogenic lines could be generated and maintained from seven out of ten trees tested using Pinto *et al.* (2002) protocol.

Multiplication

It is well known that external stimuli influence somatic embryos maturation (Gaj, 2004). Based on this, we demonstrate here that, for cork oak, somatic embryogenesis do not follow a pattern of proliferation for all genotypes assessed within the same line of time.

Mass increase and cotiledonary evolution was distinct among genotypes. While CL11 augmented the number of globular embryos, CL36 and CL8 presented an increment of cotiledonary embryos. This indicates that genotype has influence in how somatic embryos proliferate, which may affect future plant conversion. Nevertheless, since new embryos are mainly of multicellular origin, some proliferation abnormalities might occur (Puigderrajols *et al.*, 1996, 2001). As reported by Hernández *et al.* (2003a, 2003b), when these masses were broken up into small pieces and subcultured their growth rate, measured as relative fresh

weight increase, was very high and strongly influenced by genotype. Genotype also affected the number of detachable embryos produced by the embryogenic clusters.

Conversion

Somatic embryos which underwent spontaneous maturation (i.e. secondary embryogenesis ceased and cotyledons enlarged were selected for conversion. After cold treatment, one of the most common treatments for cork oak somatic embryos conversion into plantlets (e.g. Hernández *et al.*, 2003a, 2003b) the number of coordinated root and shoot growth of these embryos, defined as germination (Hartmann and Kester 1983) was recorded as well as the presence of secondary somatic embryogenesis.

Germination was often difficult to obtain, while root or shoot emergence was much more frequent. Once again genotype effect was clear. CL12 despite had roots and shoots forming, there was never synchronization. A clear recurrent problem which affected all genotypes equally was secondary embryogenesis after conversion. Pedroso and Pais (1995) reported that repetitive embryogenesis was observed in 11% of *C. japonica* embryos. However, six genotypes out of ten germinated and plants were acclimatised successfully. Chilling and maturation in PEG-supplemented media was crucial for germination (data in press). Chilling somatic embryos is a recurrent technique to induce conversion and/or germination (e.g. Hernandez *et al.,* 2003). Cold treatment as well maturation in PEG revealed to be important to induce root/shoot formation, and seem essential to trigger germination/conversion (data not shown). It is interesting to notice that cold is often reported as promoting germination in this and other species (Garcia-Martin *et al.,* 2001) even though always regarded as not good for maturation. We believe that these data support that manipulating external factors, cork oak embling conversion still may be improved at synchronization of both apex and root formation level aiming for large scale production.

Genetic variability assessment

Molecular markers (*e.g.* AFLP, microsatellites, RAPD, RFLP) had been used as reliable, powerful and quick tools in the analyses of somaclonal variation in somatic embryogenesis of both conifers and angiosperms (*e.g.* Isabel *et al.,* 1993; Heinze and Schmidt 1995; Fourré *et al.*, 1997; Helmersson *et al.* 2004; Burg *et al,.* 2007; Leal *et al.*, 2007; Lopes *et al.*, 2008) and particularly in the *Quercus* genus (Hornero *et al.*, 2001b; Sanchez *et al.*, 2003; Wilhelm *et al.*, 2005; Lopes *et al.*, 2006; Valladares *et al.*, 2006). Our study in cryopreservation of cork oak somatic embryos (Fernandes *et al.,* 2008), using AFLPs, revealed us the potential of this

technique to assess cork oak genetic stability. Once again this method was found to be reliable and very informative. When compared donor plant, somatic embryo and converted plant a clustering analyses putted together each three of each genotype. With the exception of CL2 which we found to have very high variability all the remain five genotypes showed high indexes of similarity. Moreover, despite two clear groups are formed, no relation with cork production was found.

In the specific case of somatic embryogenic lines of *Q. suber*, until the moment, either RAPD (Gallego, 1997), AFLP (Hornero *et al.,* 2001b; Fernandes *et al.*, 2008) or microsatellites (Lopes *et al.*, 2006) were used to evaluate somaclonal variation among somatic embryos within some embryogenic lines, and/or even between the explant leaves, embryos and emblings. Our laboratory has combined several genetic and molecular analyses of the somatic embryogenesis process in *Q. suber*. For instance, based on the protocol for somatic embryogenesis in *Q. suber* designed by Pinto *et al.* (2002), Loureiro *et al.* (2005) used flow cytometry and found no nDNA content nor ploidy changes, while Lopes *et al.* (2006) found only one SSR mutation in a long-term embryogenic culture derived from one adult genotype. These genetic studies are, in the present paper, complemented with AFLP analyses to further validate this cork oak SE protocol.

In our study AFLP analyses do not show significant somaclonal variation between the field tree, somatic embryos and regenerated plants by using the modified somatic embryogenic protocol developed by Pinto *et al.* (2002). Despite some reports of emblings regenerated from embryogenic callus cultures of e.g. white spruce (Isabel *et al.,* 1996), peach (Hashmi *et al.,* 1997) and date palm (Saker *et al.*, 2000) have been found to exhibit somaclonal variation, our study presents a low level of

These small differences might be due to putative small mutations, DNA methylation and selection of subpopulations (Müller *et al.,* 2007). Considering that the enzymes used for digestion are methylation-insensitive (Bonnema *et al.*, 2002), the possibility that DNA methylation could have caused these polymorphism is rather low. Nevertheless, assuming that detected polymorphisms were due to putative mutations caused by the SE procedure, the rates are, however, low. Moreover, Wilhelm *et al.* (2005), using microsatellites, found variation in embryogenic lines of *Q. robur* but not in the regenerated plantlets. Since embryogenic cultures are exposed to plant growth regulators and suffer high cellular division rates, some somaclonal variation may occur. Though, none of this putative variation was found at the emblings. This supports that the somatic embryogenic process did not induce changes in gene structure, which could significantly affect regenerated plantlets. Similar

findings have been reported for *Picea abies* (Heinze and Schmidt, 1995) and *Q, serrata* (Ishii *et al.*, 1999).

In conclusion, this work show no genetic variability in plants obtained according to the modified Pinto *et al.*, (2002) protocol, with respect to RAPD fingerprinting. Together with these results, those obtained by flow cytometry (Loureiro *et al.* 2005), microsatellites (Lopes *et al.*, 2006) and morphological characterization of somatic embryos in this species (*e.g.* Pinto *et al.*, 2002) support that our protocol used to somatic embryogenesis may be performed to provide true to type plants. We demonstrate here that controlling external conditions of cork oak maturation improves somatic embryo quality and increases further germination and conversion rates. Therefore, the SE protocol published previously (Lopes *et al.,* 2006; Pinto *et al.,* 2002) is presently improved by maturating somatic embryos in a PEG-containing medium then transferred to basal medium where they were submitted to a two months cold treatment in the dark. Moreover, we demonstrate that these conditions promote efficient germination and plants obtained are true-to-type. Successful manipulation of maturation/germination (data not shown) conditions also provides valuable tools for further studies on controlling synchronization at these stages.

References

Barro, F., Canalejo, A., Martin, A., 1999. Genomic influence on somatic embryogenesis in the *Triticeae*. Plant Cell Reports 18, 769-772.

Berlyn, G.P., Beck, R.C., Renfroe, M.H., 1986. Tissue culture and the propagation and genetic improvement of conifers. Tree Physiol 1, 227-240.

Bonnema, G., Berg, P., Lindhout, P., 2002. Aflps mark different genomic regions compared with RFLPs: a case study in tomato. Genome 45, 217-221.

Bueno, M.A., Astorga, R., Manzanera, J.A., 1992. Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiologia Plantarum 85, 30-34.

Burg, K., Helmersson, A., Bozhkov, P., von Arnold, S., 2007. Developmental and genetic variation in nuclear microsatellite stability during somatic embryogenesis in pine. J. Exp. Bot. 58, 687-698.

Cheliak, W.M., Klimaszewska, K., 1991. Genetic variation in somatic embryogenic response in open-pollinated families of black spruce. Theoretical and Applied Genetics 82, 185-190.

Coart, E., Lamote, V., De Loose, M., Van Bockstaele, E., Lootens, P., Roldan-Ruiz, I., 2002. AFLP markers demonstrate local genetic differentiation between two indigenous oak

species [*Quercus robur* L. And *Quercus petraea* (Matt.) Liebl] in Flemish populations. Theoretical and Applied Genetics 105, 431-439.

Cuenca, B., San-Jose, M., Martinez, M.T., Ballester, A., Vieitez, A.M., 1999. Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Reports 18, 538-543.

Elmaataoui, M., Espagnac, H., 1987. Neoformation of Somatic Embryos-Like Structures from Cork-Oak (*Quercus suber* L) Tissue-Cultures. Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences 304, 83-88.

FAO, 1996. Food and Agriculture Organisation. Country Report for Portugal. International Conference and Programme for Plant Genetic Resources, FAO, p. 15.

Fernandes, P., Rodriguez, E., Pinto, G., Roldan-Ruiz, I., De Loose, M., Santos, C., 2008. Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. Tree Physiology 28, 1841-1850.

Fernández-Guijarro, B., Celestino, C., Toribio, M., 1995. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell, Tissue and Organ Culture 41, 99-106.

Fourré, J.L., Berger, P., Niquet, L., André, P., 1997. Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. TAG Theoretical and Applied Genetics 94, 159-169.

Gaj, M.D., 2004. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulation 43, 27-47.

Gallego, F.J., I. Martinez, C. Celestino, M. Toribio, 1997. Testing somaclonal variation using RAPD's in *Quercus suber* L. somatic embryos. International Journal of Plant Sciences 158 No. 5, 563-567.

Garcia-Martin, G., Gonzalez-Benito, M.E., Manzanera, J.A., 2001. *Quercus suber* L. somatic embryo germination and plant conversion: Pretreatments and germination conditions. In Vitro Cellular & Developmental Biology-Plant 37, 190-198.

Hartmann, H.T.; kester, D.E. Plant propagation: principles and practices. 4.ed. New York: Englewood Clipps, 1983. 727p.

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L., Hammerschlag, F., 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Reports 16, 624-627.

Heinze, B., Schmidt, J., 1995. Monitoring genetic fidelity vs somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. Euphytica 85, 341-345.

Helmersson, A., von Arnold, S., Burg, K., Bozhkov, P.V., 2004. High stability of nuclear microsatellite loci during the early stages of somatic embryogenesis in Norway spruce. Tree Physiol 24, 1181-1186.

Hernandez, I., Celestino, C., Alegre, J., Toribio, M., 2003a. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis II. Plant regeneration from selected cork oak trees. Plant Cell Reports 21, 765-770.

Hernández, I., Celestino, C., Martinez, I., Hornero, J., Gallego, F.J., Toribio, M., 1999. Induction of somatic embryogenesis in leaves from mature *Quercus suber* L. Book of abstracts, COST 822, WG 1 'Physiology and control of plant propagation in vitro', Krakow, Poland, 7-10 October, 17-18.

Hernandez, I., Celestino, C., Toribio, M., 2003b. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Reports 21, 759-764.

Hornero, J., Gallego, F.J., Martinez, I., Toribio, M., 2001a. Testing the conservation of *Quercus* spp. Microsatellites in the cork oak, *Q. suber* L. Silvae Genetica 50, 162-167.

Hornero, J., Martinez, I., Celestino, C., Gallego, F.J., Torres, V., Toribio, M., 2001b. Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. International Journal of Plant Sciences 162, 827-833.

Isabel, N., Boivin, R., Levasseur, C., Charest, P.M., Bousquet, J., Tremblay, F.M., 1996. Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea glauca*, Pinaceae). American Journal of Botany 83, 1121-1130.

Isabel, N., Tremblay, L., Michaud, M., Tremblay, F.M., Bousquet, J., 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.) B.S.P. TAG Theoretical and Applied Genetics 86, 81-87.

Ishida, T.A., Kimura, M.T., 2003. Assessment of within-population genetic structure in *Quercus crispula* and *Quercus dentata* by amplified fragment length polymorphism analysis. Ecological Research 18, 619-623.

Ishii, K., Thakur, R., Jain, S.M., 1999. Somatic embryogenesis and evaluation of variability in somatic seedlings of *Quercus serrata* by RAPD markers. In: Mohan Jain, S.; Gupta, P. K.; Newton, R. J., eds. Somatic embryogenesis in woody plants, vol. 4. Dordrecht: Kluwer Academic Publishers 403-414.

Kaeppler, S.M., Kaeppler, H.F., Rhee, Y., 2000. Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43, 179-188.

Kim, Y., 2000. Somatic embryogenesis in *Quercus acutissima*. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer, Dordrecht, 671-686.

Kim, Y.W., Youn, Y., Noh, E.R., Kim, J.C., 1997. Somatic embryogenesis and plant regeneration from immature embryos of five families of *Quercus acutissima*. Plant Cell Reports 16, 869-873.

Larkin, P., Scowcroft, W., 1981. Somaclonal variation — a novel source of variability from cell cultures for plant improvement. TAG Theoretical and Applied Genetics 60, 197-214.

Lopes, T., Capelo, A., Brito, G., Loureiro, J., Santos, C., 2009. Genetic variability analyses of the somatic embryogenesis induction process in *Olea* spp. using nuclear microsatellites. Trees-Structure and Function 23, 29-36.

Lopes, T., Pinto, G., Loureiro, J., Costa, A., Santos, C., 2006. Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. Tree Physiology 26, 1145-1152.

Loureiro, J., Pinto, G., Lopes, T., Dolezel, J., Santos, C., 2005. Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry. Planta 221, 815-822.

Manzanera, J.A., Astorga, R., Bueno, M.A., 1993. Somatic embryo induction and germination in *Quercus suber* L. Silvae Genetica 42, 90-93.

Merkle, S.A., 1995. Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tissue Cult Biotechnol 1, 112-121.

Muller, J., Day, J.G., Harding, K., Hepperle, D., Lorenz, M., Friedl, T., 2007. Assessing genetic stability of a range of terrestrial microalgae after cryopreservation using amplified fragment length polymorphism (AFLP). American Journal of Botany 94, 799-808.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

Park, Y.S., 2002. Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Annals of Forest Science 59, 651-656.

Park, Y.S., Pond, S.E., Bonga, J.M., 1993. Initiation of somatic embryogenesis in white spruce (*Picea glauca*) – genetic control, culture treatment effects, and implications for tree breeding. Theoretical and Applied Genetics 86, 427-436.

Pedroso, M.C., Pais, M.S., 1995. Plant regeneration from embryogenic suspension-cultures of *Camellia japonica*. In Vitro Cellular & Developmental Biology-Plant 31, 31-35.

Pinto, G., Amaral, R., Santos, C., Carnide, O., 2001. Somatic embryogenesis in calluses of leaves from 3 years old *Quercus suber* L. Plants. Quality enhancement of plant production through tissue culture. In: Book of Abstracts. Second Meeting of the Cost 843 WG3. Office for Official Publications of the European Communities, Carcavelos.

Pinto, G., Valentim, H., Costa, A., Castro, S., Santos, C., 2002. Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. Tree. In Vitro Cellular & Developmental Biology-Plant 38, 569-572.

Puigderrajols, P., Fernández-Guijarro, B., Toribio, M., Molinas, M., 1996. Origin and early development of secondary embryos in *Quercus suber* L. International Journal of Plant Sciences 157, 674-684.

Puigderrajols, P., Mir, G., Molinas, M., 2001. Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in cork oak (*Quercus suber* L.). Annals of Botany 87, 179-189.

Saker, M.M., Bekheet, S.A., Taha, H.S., Fahmy, A.S., Moursy, H.A., 2000. Detection of somaclonal variations in tissue culture-derived date palm plants using isoenzyme analysis and RAPD fingerprints. Biologia Plantarum 43, 347-351.

Sanchez, M.C., Martinez, M.T., Valladares, S., Ferro, E., Vieitez, A.M., 2003. Maturation and germination of oak somatic embryos originated from leaf and stem explants: RAPD markers for genetic analysis of regenerants. Journal of Plant Physiology 160, 699-707.

Toribio, M., Celestino, C., Gallego, F.J., Martinez, I., 1998. Induction of somatic embryogenesis in tissues from mature oak trees. COST 822, WG 1 'Physiology and control of plant propagation in vitro', meeting in Lisse, The Netherlands.

Valladares, S., Sanchez, C., Martinez, M.T., Ballester, A., Vieitez, A.M., 2006. Plant regeneration through somatic embryogenesis from tissues of mature oak trees: true-to-type conformity of plantlets by RAPD analysis. Plant Cell Reports 25, 879-886.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP - a new technique for DNA-fingerprinting. Nucleic Acids Res 23, 4407-4414.

Wilhelm, E., Hristoforoglu, K., Fluch, S., Burg, K., 2005. Detection of microsatellite instability during somatic embryogenesis of oak (*Quercus robur* L.). Plant Cell Reports 23, 790-795.

Wilkinson, T.I.M., Wetten, A., Prychid, C., Fay, M.F., 2003. Suitability of cryopreservation for the long-term storage of rare and endangered plant species: a case history for *Cosmos atrosanguineus*. Ann Bot 91, 65-74.

Chapter VII

Cork oak somatic embryogenesis: concluding remarks

Conclusions

Up to date, plant regeneration in cork oak by SE has been obtained from different initial explants, in a process highly conditioned by, among other factors, the individual genotype, which has hampered a robust SE protocol with large applications. In the present work we demonstrate that cork oak is one of the few Dicotylodonea forest species in which reproducible protocols for complete plant regeneration from several selected genotypes of adult trees have been obtained. The multiplication ability of this SE process is guaranteed by the occurrence of a repetitive process that dispenses external addition of PGRs. In fact, the whole plant regeneration process can be achieved almost without the addition of PGRs. This Thesis clearly demonstrates that SE is a highly promising plant regeneration method in addition to classical approaches. One of its main advantages is the possibility to induce and regenerate true-to-type plants from selected adult trees of high commercial value. Furthermore, the existence of an optimized cryopreservation protocol for the embryogenic lines allows to preserve the embryogenic lines until the regenerated plants are tested under field conditions (Figure 21).

Hereby we describe the strategies/methodologies for establishing a collection of germplasm of elite genotypes in vitro propagation of oak (Loureiro et al. 2005; Pinto et al. 2002; Santos et al. 2007) and cryopreservation (Fernandes *et al*., 2008) already developed in our laboratory. These involve the main areas simplified in figure 21. At this point, it is possible to use our improved SE protocol (detailed described in figure 20, chapter VI) to achieve large scale propagation of cork oak.

The original SE protocol developed by Pinto *et al.* (2002) still presentd some bottlenecks, especially in the control of the repetitive SE, and on the maturation and germination/acclimatization stages of the SE process. Here we demonstrate that maturation and conversion stages may be improved by introducing new conditions such as osmotic stress and chilling. This parameters lead to reserve substance accumulation similar to those verified for zygotic embryos, thus improving converstion and enhancing the complete protocol.

However, further studies should be carried out to optimize these intermediate steps, so that the rates of plant regeneration increase to levels that fulfil the needs of the cork oak breeding and industry. I would suggest a more profound analysis of culture media nutrient composition, light and temperature improvements. These studies should be focused on

somatic embryo development and possible in order to guarantee a continuous development towards a plantlet instead repetitive somatic embryogenesis.

Future directions

The combination of biotechnology techniques described here for the cork allowed the creation in our laboratory of a small collection of germplasm, which will be extended in order to integrate the strategies of other collections in the country, to not only preserve genotypes of interest but also to select how to combine the two approaches of conservation and propagation to maximize the genetic gain. This approach will allow to:

1. Preserve/multiply "plus" genotypes currently in the field that can be reinstated.
2. Make available to producers elite genotypes previously selected and characterized, ensuring its performance in the field, given the characteristics for which the mother plant was selected (*e.g.* by the cork industry).
3. Through physiological, genetic and other biotechnology techniques, to characterize and select genotypes with better performance under adverse conditions such as temperature rise, drought or disease, crucial aspects of the strategy for forest species response to global climate change.

This integration can lead to a future National Network of cork oak germplasm, where the research should be combined with services (*e.g.* transfer of individuals to producers in accordance with the desired selected characteristics). In the research field should be highlighted improvement in trials such as: a) cork production, b) resistance to diseases, c) resistance to factors related to climatic changes such as increased temperature or water stress. It is further shown that some of these studies, particularly those related to cork response to increased temperature and water stress are already in course. Finally, it remains to note that this work underpins the importance of culture in vitro and cryopreservation as fundamental to conservation and propagation of elite plants in large scale, combining conservation of genotypes of commercial interest in maintaining biodiversity.

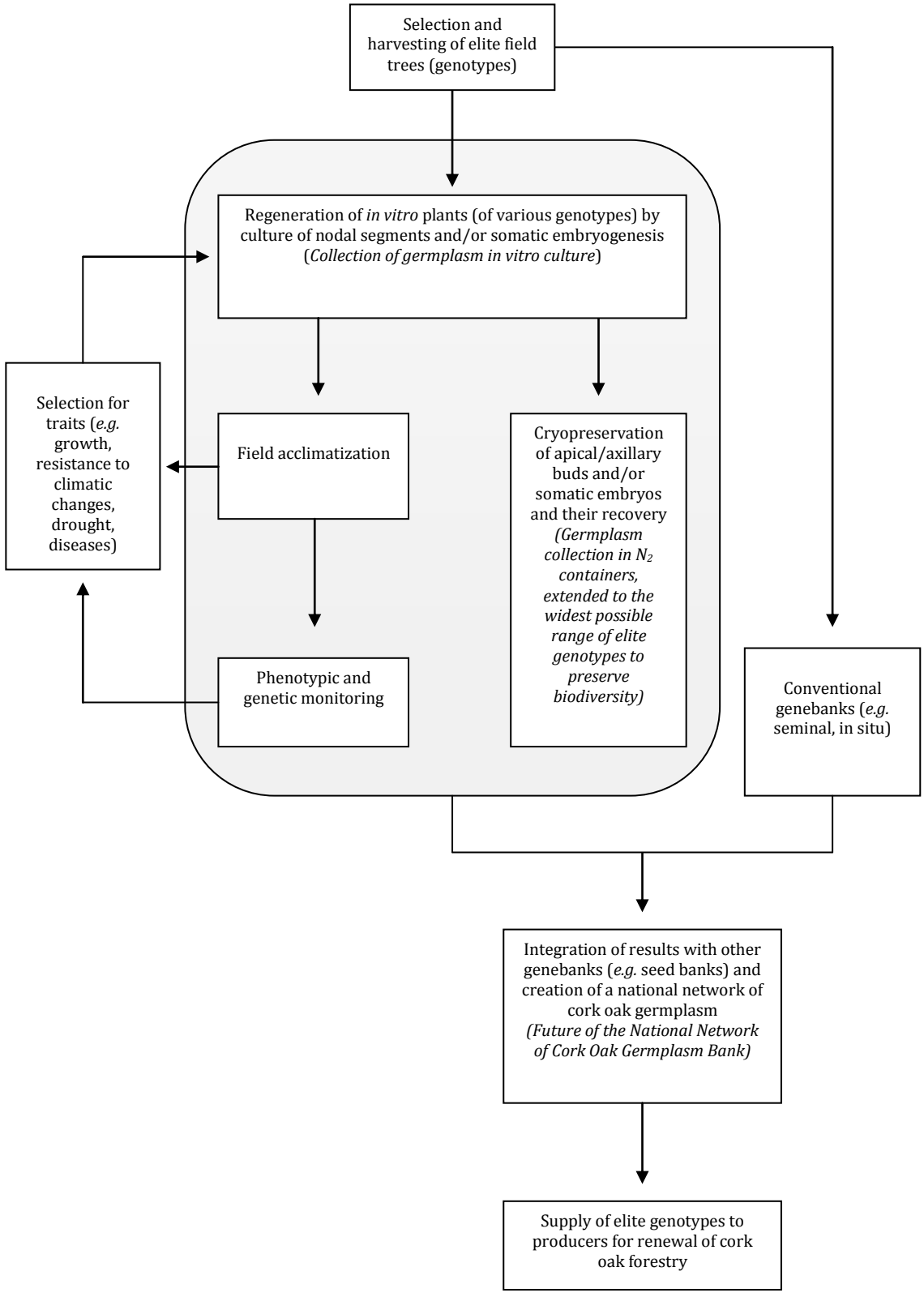

Figure 21: Main steps of combining *in vitro* propagation techniques with cryopreservation to conserve and regenerate elite genotypes of cork oak (genebank schematics).

References

Fernandes, P., Rodriguez, E., Pinto, G., Roldan-Ruiz, I., De Loose, M., Santos, C., 2008. Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. Tree Physiology 28, 1841-1850.

Loureiro, J., Pinto, G., Lopes, T., Dolezel, J., Santos, C., 2005. Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. Using flow cytometry. Planta 221, 815-822.

Pinto, G., Valentim, H., Costa, A., Castro, S., Santos, C., 2002. Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. Tree. In Vitro Cellular & Developmental Biology-Plant 38, 569-572.

Santos, C., J. Loureiro, T. Lopes and G. Pinto. 2007. Genetic fidelity of in vitro propagated cork oak (*Quercus suber* L). *In* Protocols for Micropropagation of Woody Trees and Fruits. Eds. S.M. Jain and H. Häggman. Springer-Verlag, Berlin, pp 67–84.